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A STUDY OF THE ANAEROBIC MICROFLORA OF ENSILED GRASS.

by

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C O N T E N T S

I N T R O D U C T I O N

Information on the occurrence and activities of anaerobic micro-organisms in ensiled materials may be obtained from two types of investigation : (a) bacteriological examinations and (b) chemical investigations giving data on the presence or absence of butyric acid.

Bacteriological Investigations

Serious bacteriological work on the anaerobic micro-flora of silage began when such fodder was introduced into countries engaged in the manufacture of hard cheeses, in particular Switzerland. The cheese produced was spoiled by the multiplication of an anaerobe identified with Bacillus amylobacter Bredemann (Kürsteiner, 1918). Kürsteiner (1926) showed that whereas the normal content of these organisms in cow faeces was 10 - 400 per g. the count could be increased 10,000 - 50,000 times by the feeding of silage. The whole picture was elucidated by Burri, Staub & Hohl (1919) by their demonstration of large numbers of B. amylobacter in silage, in the faeces of cows fed on silage and in the milk produced under such conditions.

These findings have been confirmed by more recent work performed by Hostettler, Sahli & Binz (1941a; 1941b). In their experiments none of the bacteria in question or only small numbers were found in the milk of cows which had received no silage. When silage was fed, the majority of milks contained butyric acid anaerobes; the counts varied from 0 to 100,000 organisms/

organisms per 100 ml. depending on the type of silage. Richard & Heinzl (1946) found that when cows were given hay, fresh grass or ensiled fodder free from B. amylobacter no increase of this organism occurred in their faeces. These authors (Richard & Heinzl, 1946) further showed that such bacteria did not multiply to any extent in the alimentary tract. It follows that B. amylobacter multiplies extensively during the fermentation in many ensiled materials. B. amylobacter Bredemann is now considered to be identical with Clostridium butyricum Prazmowski (Breed, Murray & Hitchens, 1948).

In extensive experiments Scheunert & Schieblich (1926) attempted to follow the bacteriological changes in a variety of materials conserved by ensilage. In some cases an initial heating to approximately 50°C. and in others little or no heating occurred. When they were fresh, grass, clover, lucerne, sugar-beat leaves and legume-cereal mixtures contained only small numbers of obligate anaerobes, on an average 50 per g. These authors found that during the fermentation three species of anaerobes could multiply : Bacillus putrificus Bienstock B. putrificus tenuis Zeissler, and B. amylobacter. From their description of B. putrificus as a strongly proteolytic organism, the strains in question should be considered to be identical with the type now commonly called Clostridium sporogenes and not with the weakly proteolytic terminal-sporing organism which has also been referred to as B. putrificus (Hartsell & Rettger, 1934). B. putrificus tenuis is given by Breed, Murray & Hitchens (1948) as a synonym for Clostridium bifermentans Weinberg & Seguin.

All/

All three species could reach numbers of up to 5 X 10^6 per g. of silage. B. amylobacter was found to multiply in instances where no increase of the proteolytic species had occurred, but where growth of proteolytic anaerobes took place increased counts of the saccharolytic organism were generally found. In the samples where heating had occurred during the fermentation, the anaerobic micro-organisms started to proliferate only after cooling set in. The authors arrived at the rather vague conclusion that the activity of obligate anaerobes will depend on the composition of the material to be ensiled and on the behaviour of the aerobic micro-flora.

In a further paper Schieblich (1926) pointed out that B. putrificus (= Cl. sporogenes) and B. putrificus tenuis have in his experience always been present in silage spoiled through protein breakdown and that they produce the typical foul smell often found in such samples. Proteolytic facultative aerobes did not produce such a smell.

The same author (Schieblich, 1931) investigated the effects on the final result of the moisture content of the material to be preserved. In a grass-clover mixture with a moisture content of 85% B. amylobacter reached numbers of 5 X 10^6 per g., but in the same material containing 75% and 65% water the organism failed to multiply. The addition of 1% or more of lactose or sucrose prevented the growth of the anaerobe at the high moisture content. Similar experiments were conducted with lucerne (Schieblich, 1932) but with rather different results. Lucerne containing 79% and 85% water produced a poorer silage than the same material containing 65%, but in these cases no multiplication/

multiplication of anaerobes occurred.

Ruschmann & Harder (1932) presented evidence that anaerobes may proliferate extensively in ensiled fodder, finding butyric acid bacteria up to 10^7 per g. of silage.

New ground was broken in two papers by van Beynum & Pette (1935-36, 1936). Up to this time the ability of B. amylobacter and related types to ferment lactate had not been systematically examined. This omission is rather surprising as the sole danger of silage to the hard cheese industry lies in the butyric fermentation of lactate. The two above mentioned authors investigated the problem of lactate fermentation and discovered that by no means all strains of butyric acid bacteria could attack lactates. They separated their large collection of strains into two distinct species : (a) Clostridium saccharbutyricum which utilises a wide range of carbohydrates but not lactate and (b) Clostridium tyrobutyricum which ferments lactate and of the sugars only glucose and fructose and in the case of a few strains galactose. They further showed that both these types occurred in silage.

These findings have, however, been challenged. In studying anaerobes isolated from fodder preserved by the A.I.V. and other methods, Burri (1936) could distinguish strains intermediate between these species in fermentative ability. He nevertheless isolated cultures which could not attack lactates. More recently Richard (1948; 1950) came to the conclusion that almost all strains of Clostridium butyricum and related bacteria could attack lactate.

Van Beynum & Pette (1936) also investigated the lower pH limits/

limits for the growth of their strains of butyric acid anaerobes. None grew below pH 4.0; a few strains initiated multiplication at pH 4.24 but this was not detectable until after three months' incubation. On examining a large number of A.I.V. silages made under farm conditions, they found that almost all contained butyric acid bacteria and butyric acid although the pH level of the fodder appeared to be 4.0 or less. The apparent contradiction was explained by the realisation that under farm conditions the conserved material is not homogeneous, especially if additions such as acid or molasses are made. Pockets remain into which acids slowly or never penetrate.

A further point is brought out by van Beynum & Pette. In some samples of silage investigated large numbers of bacteria capable of producing lactic acid were present, but only very small amounts of lactic acid could be detected chemically. In these samples butyric acid producing organisms had flourished and were presumed to have converted the lactic to butyric acid.

Work on the number of spores of obligate anaerobes on fresh grass and in ensiled grass at various stages of the fermentation process was published by Allen & Harrison (1937a). Fresh grass, taken from plots which had been recently grazed, not grazed for several months, exposed to weather conditions or covered, gave low and similar counts (100 per g. or less). On ensiling, the count remained low for the first few days and then increased to approximately 10^6 per g. at 10 to 15 days. It either remained at this figure or increased to 10^8 per g. during the next few weeks and then slowly declined. Only Cl. sporogenes was isolated when spring grass was preserved.

In/

In autumn grass to which various additions had been made and which had a higher moisture content, Cl. sporogenes, Cl. butyricum and Cl. welchii were found in approximately equal numbers.

Discussion of results

The papers reviewed above represent the main bacteriological contributions to this subject. The information they supply may be evaluated by listing the essentials of a thorough knowledge of the function of anaerobic micro-organisms in silage. The following questions require to be answered : 1) Do anaerobes multiply in silage and if multiplication takes place does it do so frequently or only under exceptional conditions? 2) What species are of importance? 3) How extensive is the multiplication and at what stage of the fermentation does it take place? 4) What factors influence the development of such organisms? 5) What changes do they catalyse in the fodder? Some of the answers will be inter-related but for the sake of simplicity they will be considered separately.

(i) Occurrence of growth. All workers have found high numbers of anaerobes in a proportion of the samples examined and these numbers have been much higher than those occurring on fresh material.

Van Beynum & Pette (1936) discovered butyric acid producing anaerobes in almost all of the many silages they investigated. In Switzerland it has been found impossible to produce hard cheeses from the milk of cows fed on silage. In that country legislation has even been introduced to prevent such/

such milk being sent to cheese factories, (Ruschmann & Harder, 1932). Scheunert & Schieblich (1926), Schieblich (1926) and Allen & Harrison (1937a) provided some evidence that proteolytic species of the genus Clostridium also have a wide distribution in ensiled fodder. The conclusion may therefore be drawn that obligate anaerobes do proliferate in ensiled materials and that some species at least do so frequently.

(ii) Species of importance. Cl. butyricum or related types, Cl. sporogenes, Cl. bifermentans and Cl. welchii have been shown to initiate growth in ensiled fodder while other types have not been found to multiply. But the attention of most workers has been directed, not to discovering what types of anaerobes were present in a certain sample, but to the isolation of a particular species viz. Cl. butyricum or related bacteria. The methods used in such cases were designed for the elimination, as far as possible, of other micro-organisms e.g. van Beynum & Pette (1935 - 36). In two investigations a more general approach was adopted, namely those of Allen & Harrison (1937a) and Scheunert & Schieblich (1926). The first mentioned authors, in order to simplify the work, employed a heat-treatment sufficient to kill vegetative cells. Anaerobes which do not form spores are well known and these, if they proliferate in silage, would have been missed. Scheunert & Schieblich worked with unheated as well as heated material, but the media they used - milk and glucose-bouillon - would permit good growth of the many types of facultative anaerobes present in ensiled material. Obligate anaerobes occurring in the original

inoculum/

inoculum could have been suppressed and therefore missed under such conditions.

A great deal of work had to be undertaken before reliable methods could be evolved for the detection of various types of strict anaerobes in other habitats where many species of obligate and facultative anaerobes flourish together, such as gangrenous infections and the rumen of cattle. In view of the paucity of work on the anaerobes of silage and of the other considerations set out above, it would appear that while the literature provides some guidance on the relative importance of different species, a final answer has not yet been supplied.

(iii) Extent of growth and the time of its occurrence.

Figures for the numbers attained by butyric acid-forming anaerobes are quoted by most of the papers reviewed, but only in two (Allen & Harrison, 1937; Scheunert & Schieblich, 1926) has an attempt been made to ennumerate other anaerobes or to determine at what stage of the fermentation multiplication takes place. Further, an examination of the methods employed shows that they were inadequate for tracing the development of the organisms.

In all instances except two, (Scheunert & Schieblich 1926; Ruschmann & Harder, 1932) pasteurisations of inocula were carried out in order to eliminate non-spore-forming bacteria. Spores are not formed during active growth, the phase of most active metabolism (Knaysi, 1949). Therefore, by counting spores entirely misleading results may be obtained.

Sporulation/

Sporulation may be greatly affected by many factors (e.g. Knaysi, 1948), but even under good conditions not every cell develops into the resistant resting stage. Torrey, Kahn & Sahlinger (1930) achieved 50 to 80% sporulation with Cl. welchii, while the best figures noted by Kaplan & Williams (1941) during investigations on Cl. sporogenes averaged 50%. Dorner (1924) observed the appearance of endospores in 50% of cells of Cl. butyricum.

One of the factors affecting sporeformation is the pH level of the medium in which the cells are growing. Cl. welchii and Cl. sporogenes develop no spores if the pH is, in the case of the former species, below 6.6 (Torrey, Kahn & Sahlinger, 1930), and, in the case of the latter, below 6.1 (Kaplan & Williams, 1941). Svartz (1932) could not induce sporeformation in strains of Cl. butyricum if the initial pH was below 6.6.

The pH levels observed even in badly preserved silage are generally much below 6.0 i.e. in the range where sporeformation by many members of the genus Clostridium is poor or does not occur. Some findings tending to contradict this point of view have however been presented by Richard (1946). While agreeing that Cl. butyricum produces endospores in peptone and bouillon media only if these are nearly neutral in reaction, he states that in a green pea-glucose mash spores were formed at all pH levels at which growth took place. The percentage of cells forming spores with the higher concentrations of hydrogen ions was not given. The explanation advanced by the author is that the ratio of available nitrogen to available carbohydrate/

carbohydrate in the pea mash was much smaller than in the other media tested. If this is the true explanation, a great variation may be expected in the percentage of cells developing into the resistant resting stage in ensiled fodder of differing protein content.

Convincing evidence concerning the accuracy with which spore counts represent the numbers of anaerobes in ensiled crops would be provided by a comparison of counts from heated and unheated portions of the same material. Some such figures are available. Among others, the following are given by Scheunert & Schieblich (1926): 5×10^6 vegetative cells of Cl. butyricum and less than 10 spores per g.; 5×10^6 vegetative cells of Cl. butyricum and 5×10^3 spores per g.; 5×10^5 vegetative cells of Cl. sporogenes and 5×10^4 spores per g.; 5×10^5 vegetative cells of Cl. bifermentans and 5×10^4 spores per g. Olsen (1951), in an investigation of silage made from sugar beet pulp, found that the numbers of vegetative cells of Cl. butyricum ranged from 10^7 to 10^{10} per g. of silage during the first 69 days after ensiling, while spore counts never exceeded 10^4 per g. After 69 days the spore count was 10,000 times lower than the actual number of organisms present. It may be concluded that spore counts have little value. They yield no information on the time when multiplication occurred and the available evidence indicates that a true picture of numbers is also not obtained.

With one exception (Olsen, 1951) in all the investigations reviewed, including those where no pasteurisation was employed, the media chosen for the cultivation of the butyric type of anaerobe constituted/

constituted a source of error. The media employed were glucose - peptone media, (in the case of Allen & Harrison (1937a) glucose - peptone + yeast extract) milk, and media containing no combined nitrogen and potato-mash.

McClung & McCoy (1934) and McClung (1940) showed that Cl. butyricum and related bacteria give counts 100 or more times higher in certain media than they do in glucose - peptone and nitrogen free media or in milk. Experiments conducted by Ritter (1932) suggest that potato-mash as used by Ruschmann & Harder (1932) is not superior in this respect to glucose - pepton agar. Dorner (1924) compared counts of vegetative cells and spores of Cl. butyricum obtained by a microscopical method with counts obtained by inoculation into glucose - peptone agar and came to the conclusion that less than 5% of the organisms formed colonies in this medium. His findings support the contention of McClung & McCoy (1934) that such media are not suitable for the enumeration of butyric acid anaerobes. van Beynum & Pette (1935 - 36) have pointed out that Cl. tyrobutyricum, a species found by them to be of common occurrence in ensiled fodder, does not ferment lactose and has little ability to grow in milk.

The position may be summed up as follows. In the majority of investigations, the numbers of one type of anaerobe at one particular stage of the fermentation have been determined and the figures obtained are of doubtful validity due to the media employed or to the heating applied or to both of these factors. In two investigations (Scheunert & Schieblich, 1926; Allen & Harrison, 1937a) where more information was sought, the results/

results are affected by the unsuitability of media in the case of the former and by the employment of a heat-treatment and unsuitable media in the case of the latter investigation.

(iv) Factors influencing the development of anaerobes. No systematic investigation of the factors controlling the growth of anaerobes in silage has been reported. Experiments which have been recorded may be critisized on the grounds of the methods employed. Considerable information is, however, available in the case of one particular factor, namely the pH level in the ensiled fodder.

The extensive chemical literature on silage consistently suggests that anaerobes are unable to develop if large amounts of acid are present and results obtained by bacteriological methods have corroborated and extended these findings. Anaerobic micro-organisms isolated from silage have been tested for their ability to multiply at various pH values (Virtanen, 1934; van Beynum & Pette, 1936). Further, cultures from silage have been identified with well-known species whose characteristics have been studied in other connections. In the case of anaerobes suspected of being active in silage the lowest pH at which growth has been found to occur is slightly above 4.0. By applying these results to their work on silages made under farm conditions, van Beynum & Pette (1936) were able to demonstrate the importance of homogeneity in ensiled fodder. Richard (1946) showed that the undissociated molecules of organic acids have an inhibitory effect on the growth of Cl. butyricum and that organic acids therefore prevent the multiplication of this species at higher

pH/

pH levels than inorganic acids. Provided the ensiled material is homogeneous and organic acids are present, control of Cl. butyricum may be obtained at pH values appreciably higher than 4.0.

(v) Changes produced by anaerobes in silage. If the changes which anaerobes initiate when multiplying in pure culture can be taken to be similar to those occurring when they proliferate in silage, the literature provides some information on this aspect of the problem. Organisms from silage have been identified with species whose metabolism has been investigated. Cl. sporogenes actively hydrolyses proteins and peptides to amino acids which are further attacked; NH_3, H_2S, H_2 and unidentified compounds having a foul smell are liberated. Cl. bifermentans catalyses similar reactions. Cl. welchii ferments a range of carbohydrates and as a result of this fermentation butyric and other organic acids accumulate. This species has also the ability to hydrolyse peptides and deaminate amino-acids (Bulloch, Bullock, Douglas, Henry, McIntosh, O'Brien, Robertson & Wolf, 1919; Breed, Murray & Hitchens, 1948; Stephenson, 1949). Bacteria of the Cl. butyricum group attack carbohydrates and lactate, the endproducts of their metabolism being H_2, CO_2 and volatile compounds in particular butyric acid (McCoy, Fred, Peterson & Hastings, 1930; Langlyke, Peterson & McCoy, 1935; van Beynum & Pette, 1935 - 36; Bhat & Barker, 1947).

Most of these changes are regarded as detrimental to the quality of silage. The simple nitrogenous compounds formed cannot be used as efficiently by animals as the more complex substances/

substances from which they were derived and their production hinders the lowering of the pH level. The formation of butyric acid, a weak acid, from lactates or from carbohydrates which might have been utilised for the production of lactic acid hinders the lowering of the pH level.

No conclusion can yet be drawn regarding the constituents of the fodder from which the butyric acid in silage is formed. Van Beynum & Pette (1935 - 36) and Burri (1936) produced evidence that certain members of the Cl. butyricum group could attack lactate and that others could not. Van Beynum & Pette (1936) further demonstrated that in some instances most of the butyric acid in ensiled crops originates from lactates but they also found that multiplication of butyric acid anaerobes which could not attack lactate occurred in ensiled grass. It appeared that both carbohydrates and lactate may be substrates. Later Bhat & Barker (1947) showed that previously used methods of demonstrating lactate attacking powers in Cl. butyricum were not reliable and Richard (1948, 1950) has come to the conclusion that almost all strains of this species ferment lactates. On the evidence available it seems probable that the greater part of the butyric acid in silage is derived from lactates.

Chemical Investigations

Butyric acid has not been found among the constituents of growing plants (Stiles & Leach, 1936; Watson, 1939; Turner, 1951). It does, however, appear in many cases when plants are ensiled. Its appearance must be due either to the respiration/

respiration of the plant cells or to the metabolism of micro-organisms.

None of the investigations described in two recent reviews of work on the respiration of plants (Stiles & Leach, 1936; Turner, 1951) have succeeded in detecting even traces of butyric acid as an endproduct of anaerobic respiration. It may be concluded that if butyric acid is formed by plant cells, the amounts produced must be very small and not at all comparable to the concentrations occurring in silage.

The only species of micro-organisms so far known which form more than minute quantities of butyric acid during the course of metabolism are obligate anaerobes (McCoy, Fred, Peterson & Hastings, 1930; Stephenson, 1949). Large numbers of such anaerobes have been discovered in ensiled fodder, and in the samples investigated bacteriologically their numbers have shown some agreement with the butyric acid content (Rushhmann & Harder, 1932; van Beynum & Pette, 1936). It seems, therefore, legitimate to assume that the butyric acid formed in silage is a metabolic product of anaerobic bacteria.

The wide-spread occurrence of butyric acid in silage (e.g. Watson, 1939) is so well known that it needs no further discussion. It confirms and extends the opinion that butyric acid producing anaerobes commonly multiply in such material. A wealth of data (reviewed by Watson, (1939) shows that the formation of butyric acid is inhibited if the pH in the ensiled fodder falls to 4.0 or less.

The effect of various factors such as the addition to fodder/

fodder before ensilage of carbohydrates, of lactobacilli and of organic and inorganic acids has been the subject of extensive research and the results have been reviewed by Watson (1939). These additions prevent the production of butyric acid because they assist in the attainment of low pH levels. Temperatures in the ensiled material of above 50°C have been shown to control butyric acid formation (Watson 1939).

Observations relating the moisture content of silage to the amount of butyric acid formed have been reported. When the moisture content of the fodder is reduced by wilting or other methods, less butyric acid tends to be formed during the fermentation (Krauss, 1941; Hellberg, 1945; Shepherd, 1948; Wilson, 1948). On the other hand, when the moisture content of the plant material is unusually high, e.g. through faulty drainage of the silo, a specific stimulation of butyric acid anaerobes appears to take place (Crasemann & Heinzl, 1949). The findings of Schieblich (1931) show some agreement with these results.

Examinations of the losses taking place during the preservation have been made and are reviewed by Watson (1939). The results show that as the final pH value of the silages increases, so do the losses. It has already been pointed out (p. 14) that the proliferation of butyric acid anaerobes will hinder the lowering of the pH level and it would therefore appear that these organisms are not merely incidental members of an undesirable microflora, but important causal agents of deterioration.

The/

The information dealing with the proliferation of anaerobic micro-organisms in ensiled materials, which can be gained from the bacteriological and chemical investigations reviewed above, contains many gaps. One of the major factors which has hindered a better understanding of the role played by anaerobes is the lack of bacteriological methods suitable for the enumeration of the dominant species. The work to be described in the following pages consists of attempts to improve methods of enumeration and of the application of the techniques which were developed to the examination of ensiled grass.

E X P E R I M E N T A L

Part I.

The Development of Methods for the Quantitative Enumeration of Vegetative Cells and Spores of Anaerobes

Objects

The aim of this part of the work has been to develop methods for the enumeration of vegetative cells as well as spores of the dominant species of anaerobes in ensiled grass.

In order to achieve this goal it was thought necessary to obtain further information regarding the identity of the types which may reach high numbers and to devise selective media for them. Two properties of the methods were deemed to be essential : (a) ability to permit the initiation of growth from small inocula and (b) sufficient simplicity to allow examinations to be made at short intervals.

The Preparation of Dilutions

When numbers of anaerobic micro-organisms are estimated by cultural methods requiring the preparation of dilutions, the death of vegetative cells during the dilution procedure may be a source of error. For anaerobes of the rumen it has been found necessary to employ very strict precautions against contact of the cells with oxygen during the preparation of dilutions (Hungate 1947).

In order to explore the position with regard to species which are likely to occur in silage, a point on which little guidance can be obtained from the literature, dilution counts were made using pure cultures of Cl. butyricum. For the preparation of cultures of this organism and the detection of viable cells in the dilutions, a slightly modified form of the corn-liver mash medium of McClung & McCoy (1934) was employed. The modification consisted of the substitution of 1.6% Oxoid dessicated liver and 0.4% Oxoid liver extract for the 1 - 2% finely ground, dried liver of the original medium. Corn-liver mash has been shown (by McClung & McCoy) to permit initiation of growth from small numbers of cells of Cl. butyricum.

In the mash only insignificant numbers of spores were formed, as was shown by dilution counts made from cultures heated at 80°C for 10 minutes. Cultures incubated for 2 or 5 days at 37°C never contained more than 100 endospores per ml. In the tests described below, decimal dilutions of cultures incubated for 24 hours at 37°C were prepared by adding 0.5 ml. to 4.5 ml. dilution fluid in 6 x $\frac{3}{4}$" test-tubes. No attempt was made/

TABLE 1.

Survival of vegetative cells of Cl. butyricum in dilution fluids

Culture	Dilution Fluid	Time of Standing after dilution	End points of dilution counts corn-liver mash[1]						
			$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$	$1/10^7$	$1/10^8$	$1/10^9$
a	Water	0	+++	+++	+++	+++	+--	---	---
	"	50 min.	+++	+++	+++	---	---	---	
b	Water	30 min.	+++	+++	+++	+++	+++	+--	---
	"	150 min.	+++	+++	+++	+++	+--	---	
c	Water	240 min.	+++	+++	+++	+--	+--	---	
d	Water	300 min.	+++	+++	+--	---	---		
e	Water	30 min.	+++	+++	+++	+++	+++	+--	---
	0.5% L.E.[2]		+++	+++	+++	+++	+++	+--	---
	1% glucose[3]		+++	+++	+++	+++	+++	+--	---
f	Water	240 min.	+++	+++	+--	+--	---	---	---
	0.5% L.E.[2]		+++	+++	+++	+++	+++	++-	---
	1% glucose[3]		+++	+++	+++	+++	++-	---	---
g	Water	300 min.	+++	+++	+--	---	---	---	
	0.5% L.E.[2]		+++	+++	+++	+++	++-	+--	---
	1% glucose[3]		+++	+++	+--	---	---	---	

1 - 3 replicates inoculated from each dilution
2 - Oxoid liver extraction in aqueous solution
3 - aqueous solution

made to use freshly autoclaved dilution fluids or to remove dissolved oxygen by heating at 100°C. Mixing of dilutions was carried out by alternately drawing liquid into and expelling it from a 1 ml. pipette. 0.5 ml. portions were transferred, either immediately after preparation of the dilutions or after standing at room temperature, to corn-liver mash, three replicate tubes being inoculated from each dilution.

Scattered statements have been discovered in the literature which indicate that certain materials have the ability to protect vegetative cells of anaerobes from the harmful action of oxygen. For example, Davies & Stephenson (1941) found that centrifuged cells of Cl. acetobutylicum lost all activity when washed and re-suspended in buffer solution, but that each of the constituents of a medium suitable for the growth of this species had some ability to prevent inactivation when added to the buffer. On the basis of these reports, a 0.5% w/v aqueous solution of liver extract (Oxoid) and a 1% w/v aqueous solution of glucose were employed, besides sterile water, as dilution fluids in the trials.

The results obtained are presented in Table I. and show (a) that vegetative cells of Cl. butyricum retain their viability for considerable periods under the conditions of the tests and (b) that after prolonged exposure to air survival was very much better in water plus liver extract and slightly better in water plus glucose than in water alone, although no difference could be detected after thirty minutes exposure.

Evidence agreeing with conclusion (a) set out above was found/

found in experiments reported by Dorner (1924). Dorner showed that/counts of vegetative cells of Cl. butyricum did not decrease during an exposure of 40 minutes to atmospheric oxygen, the cells being contained in small drops of water placed on coverglasses. After one hour, a definite drop in the count appeared, but even under these severe conditions, viable cells were present after an exposure of three hours.

Since the tests described above were of an exploratory nature and were conducted with only one of the species which may be expected to proliferate in silage, no definite conclusion can be drawn. The indications are, however, that, employing water as the dilution fluid and taking no special precautions to prevent the contact of cells with oxygen, the death of vegetative cells during the dilution procedure is not likely to be an important factor in the enumeration of anaerobes in ensiled materials. In several instances in the later stages of the work, water containing 0.5% liver extract was employed as the diluent in order to minimise still further any chances of error.

Methods of Achieving Anaerobiosis

Many methods have been shown to be successful in the cultivation of anaerobes (McIntosh & Fildes, 1917; Burri, Staub & Hohl, 1919; Hall, 1929; Zeissler, 1929; McClung, McCoy & Fred, 1935; Spray, 1936; Rosenthal, 1937; Brewer, 1940, 1942a, 1942b), but the majority of these suffer from serious drawbacks, such as being time-consuming, space-consuming or requiring special apparatus/

apparatus. The technique which would appear to possess most advantages, except for the isolation of pure cultures, is the use of reducing agents in semi-solid or solid media (Spray, 1936; Reed & Orr, 1943; Foley & Schaub, 1944; Vera, 1944a, 1944b).

Although the value of reducing agents has been well established, little information is available on two aspects of their use viz. (a) ability of the different types to prevent re-oxidation of media and (b) their toxicity to different species of anaerobes. In order to employ reducing agents most effectively these points were investigated and further, the possibility of improving by their use methods for the isolation of pure cultures was explored.

Composition of media employed

Section 1 - Ability to prevent the oxidation of media.

Methylene blue agar: Added per litre of tap water were 5g. peptone (Bacto), 30 mg. methylene blue and, except during preliminary trials on the effect of differing concentrations of agar, 15g. agar. The medium was dispensed in 7 ml. quantities in 6 x $\frac{5}{8}$ in. tubes.

Section 2 - Toxicity to anaerobes.

Semi-solid medium: Added per litre of tap water were 5g. peptone (Bacto), 5g. liver extract (Oxoid), 5g. glucose, and 2g. agar. The pH was adjusted to 5.9 - 6.0 when intended for the cultivation of Cl. butyricum and Cl. acetobutylicum and to 7.0 - 7.1 when intended for Cl. welchii and Cl. sporogenes. The semi-solid agar was dispensed in 5 ml. quantities in 5 x 0.5 in. tubes.

Section/

Section 3 - Methods for the isolation of pure cultures.

Lactate agar: Constituents per litre of tap water were 10g. peptone (Evans), 10 ml. yeast autolysate (Barker & Beck, 1942), 0.1 µg. biotin, 100 µg. para-amino-benzoic acid, 14.3 ml. sodium lactate (70% syrup), 8g. sodium acetate, 2g. cysteine hydrochloride, 0.5 ml. thioglycollic acid, 5 mg. resazurin, 15g. agar and with the pH adjusted to 6.5.

Peptone-glucose agar: Constituents per litre of tap water were 5g. peptone (Evans), 5g. liver extract (Oxoid), 10 ml. yeast autolysate (Barker & Beck, 1942), 5g. glucose, 5 mg. resazurin, 2g. cysteine hydrochloride, 0.5 ml. thioglycollic acid, 15g. agar and with the pH adjusted to 7.0.

Covering agar: Constitutents per litre of tap water were 2g. cysteine, 1 ml. thioglycollic acid, 5 mg. resazurin, 15g. agar and with the pH adjusted to 7.0.

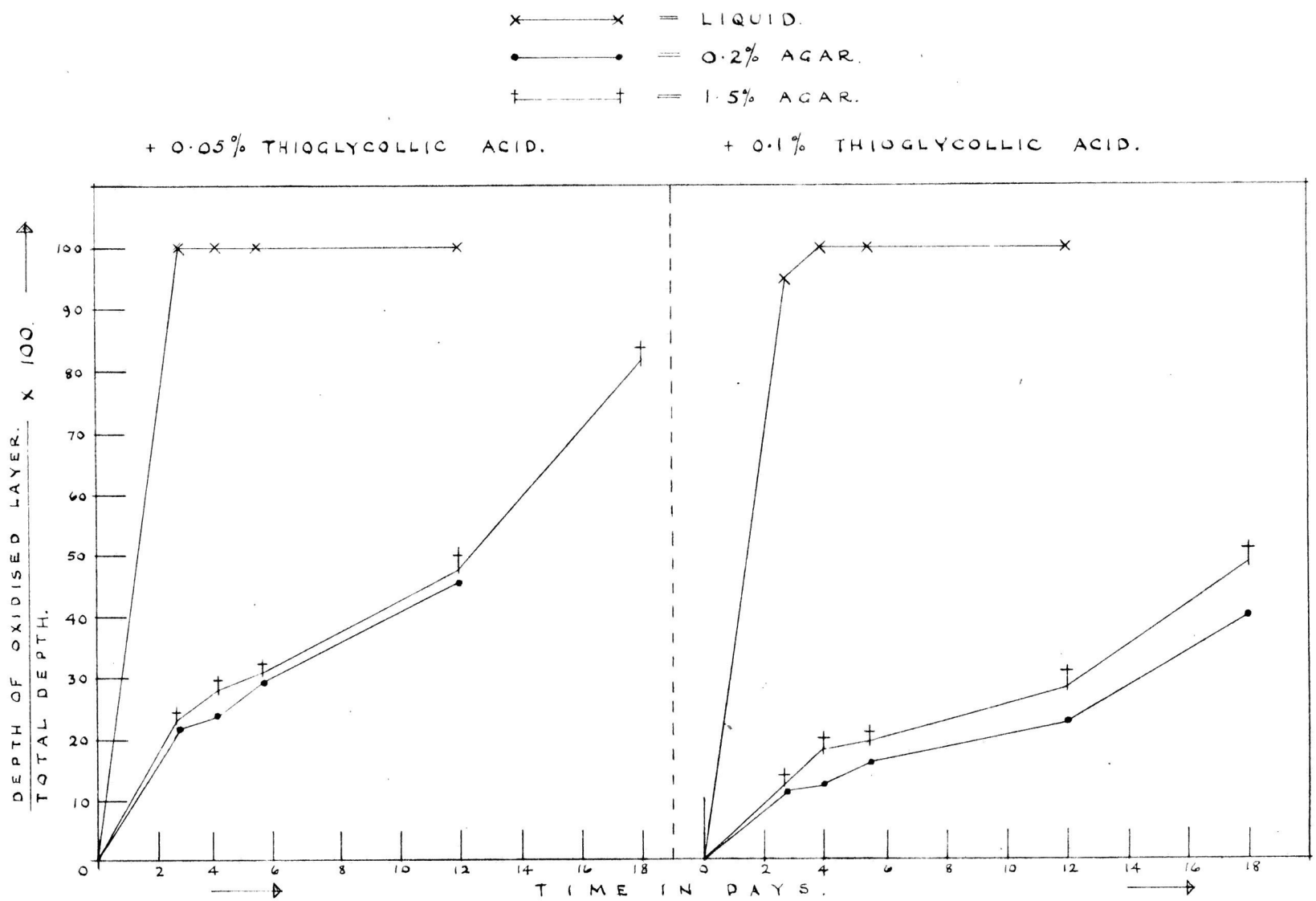
Tomato agar: Constituents per litre of medium were 400 ml. tomato juice, 10g. peptone (Evans), 5 ml. thioglycollic acid, 15g. agar, tap water to 16, and with the pH adjusted to 6.0.

Liver-extract-casein agar: Constituents per litre of tap water were 5g. liver extract (Oxoid), 5g. peptone (Evans), 20g. casein (soluble), 5 ml. thioglycollic acid, 1g. ascorbic acid, 15g. agar and with the pH adjusted to 7.0. The casein was autoclaved separately in 10% solution and added to the sterilized medium with aseptic precautions.

1. Ability to prevent oxidation of media

The reducing agents tested were thioglycollic acid, cysteine hydrochloride, sodium formaldehyde sulphoxylate, ascorbic/

FIG. 1. RATE OF OXIDATION OF LIQUID, SEMI-SOLID AND SOLID MEDIA CONTAINING THIOGLYCOLLIC ACID AND TUBED IN 6 × 5/8 IN. TUBES.



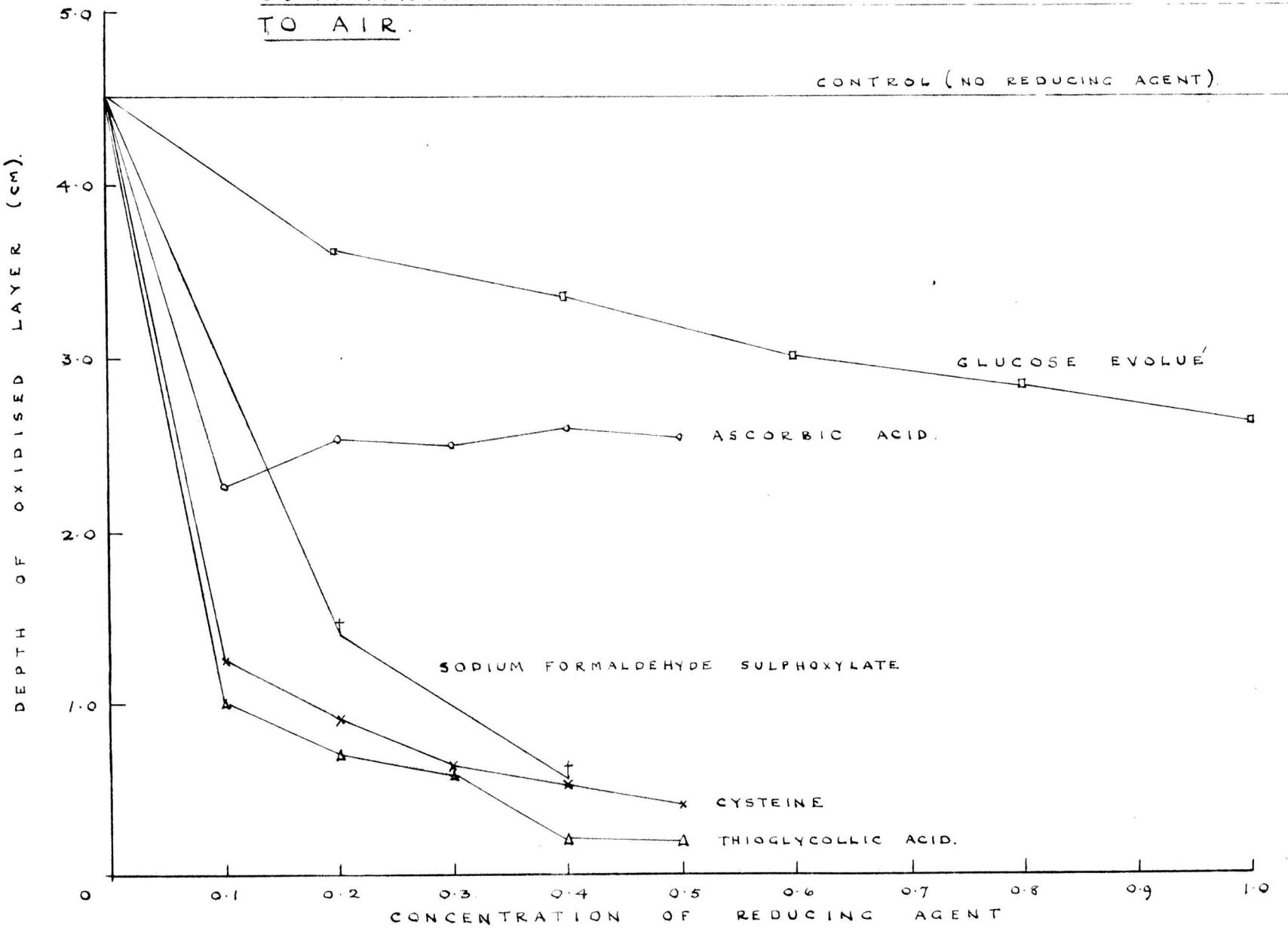
ascorbic acid (added as an aqueous solution of 'Redoxon' tablets), and glucose evolué (prepared as described by Knight, (1941)). The medium to which the reducing agents were added was a methylene blue agar described in the previous section (p. 22).

Thioglycollic acid, cysteine and sodium formaldehyde sulphoxylate were added before sterilisation of the medium at 22½ lbs. pressure momentarily in the autoclave, the pH being adjusted where necessary to 7.0. Additions of ascorbic acid, sterilised by Seitz filtration and with the pH adjusted to 7.0, and of glucose evolué were made to the sterile medium. In all cases undue agitation of media was avoided and these were solidified immediately after sterilization or the addition of the reducing agent by immersion in cold water. The progress of oxidation was followed through measurements of the depth of the layer containing oxidised methylene blue. Duplicate tubes were prepared in all the tests and the average taken as the result.

In the absence of agar, using media containing two different concentrations of thioglycollic acid, the rate of oxidation was found to be much more rapid than in the presence of 0.2% ⩒ or 1.5% ⩒ agar (Fig. 1). With 0.2% and 1.5% agar oxidation proceeded at similar rates (Fig. 1).

Tables illustrating the penetration of oxygen into the solid medium when it contained different concentrations of the five reducing agents are given in full in the appendix (p.101) and are presented in a condensed form, by a comparison of the extent/

FIG 2. PENETRATION OF OXYGEN INTO TUBED AGAR MEDIA CONTAINING REDUCING AGENTS AFTER 5 DAYS EXPOSURE TO AIR.



extent of oxidation after 5 days, in Fig. 2. Arranged in order of efficiency, thioglycollic acid, cysteine, and sodium formaldehyde sulphoxylate were the most active in preventing the ingress of oxygen. Ascorbic acid at a concentration of 0.1% w/v was comparable in efficiency to sodium formaldehyde sulphoxylate, but, in contrast to the other compounds tested, increases in the concentration of ascorbic acid did not result in increased ability to prevent oxidation. No explanation can be offered for this finding, unless the use of an impure commercial preparation has been responsible. Since ascorbic acid cannot be subjected to heat sterilisation, its use is time-consuming, and this substance and glucose evolué, the least active of the compounds tested, were regarded as the least useful of the five reducing agents.

2. Toxicity to anaerobes

To determine the effect of thioglycollic acid, cysteine, and sodium formaldehyde sulphoxylate on the initiation of growth by small inocula of four species of anaerobes viz. Cl. butyricum, Cl. acetobutylicum, Cl. welchii and Cl. sporogenes, the following tests were carried out. Decimal dilutions of corn-liver mash cultures, incubated for 24 hours at 37°C, were prepared, using water as a diluent, and the dilutions inoculated, in triplicate, into a clear semi-solid medium (p.22) to which differing concentrations of the reducing agents had been added. The medium containing 0.05% v/v thioglycollic acid served as the standard treatment against which the growth occurring with other concentrations/

TABLE 2.

Comparison of dilution counts[1] in corn-liver mash, meat infusion[2] and coagulated blood[3] media using a pure culture of Cl. sporogenes

Medium	End points of dilution counts						
	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$	$1/10^7$	$1/10^8$	$1/10^9$
Corn-liver mash	+++	+++	+++	+++	+++	+--	---
Meat infusion	+++	+++	+++	+++	+++	+++	---
Coagulated blood	+++	+++	+++	+++	+++	+++	---

1. Decimal dilutions prepared in water; three replicates inoculated from each dilution

2. Cunningham (1947)

3. Pullar (1936)

TABLE 3.

Comparison of dilution counts[1] in a peptone-liver extract-yeast extract semi-solid medium with counts in corn-liver mash or coagulated blood

Organism	Medium	End-points of dilution counts						
		$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$	$1/10^7$	$1/10^8$	$1/10^9$
Cl. butyricum	Corn-liver mash	+++	+++	++-	+--	+--	---	---
	Semi-solid medium	+++	+++	+++	+++	++-	---	---
Cl. acetobutylicum	Corn-liver mash	+++	++-	---	---	---	---	---
	Semi-solid medium	+++	+++	---	---	---	---	---
Cl. sporogenes	Coagulated blood	+++	+++	+++	+++	+++	---	---
	Semi-solid medium	+++	+++	+++	+++	+--	---	---
Cl. welchii	Corn-liver mash	+++	+++	+++	++-	---	---	---
	Semi-solid medium	+++	+++	+++	+++	++-	---	---

1. Decimal dilutions prepared in water; three replicates inoculated from each dilution.

FIG 3 EFFECT OF CYSTEINE ON THE INITIATION OF GROWTH: RESULTS OF DILUTION COUNTS (3 REPLICATES) EXPRESSED AS MOST PROBABLE NUMBERS.

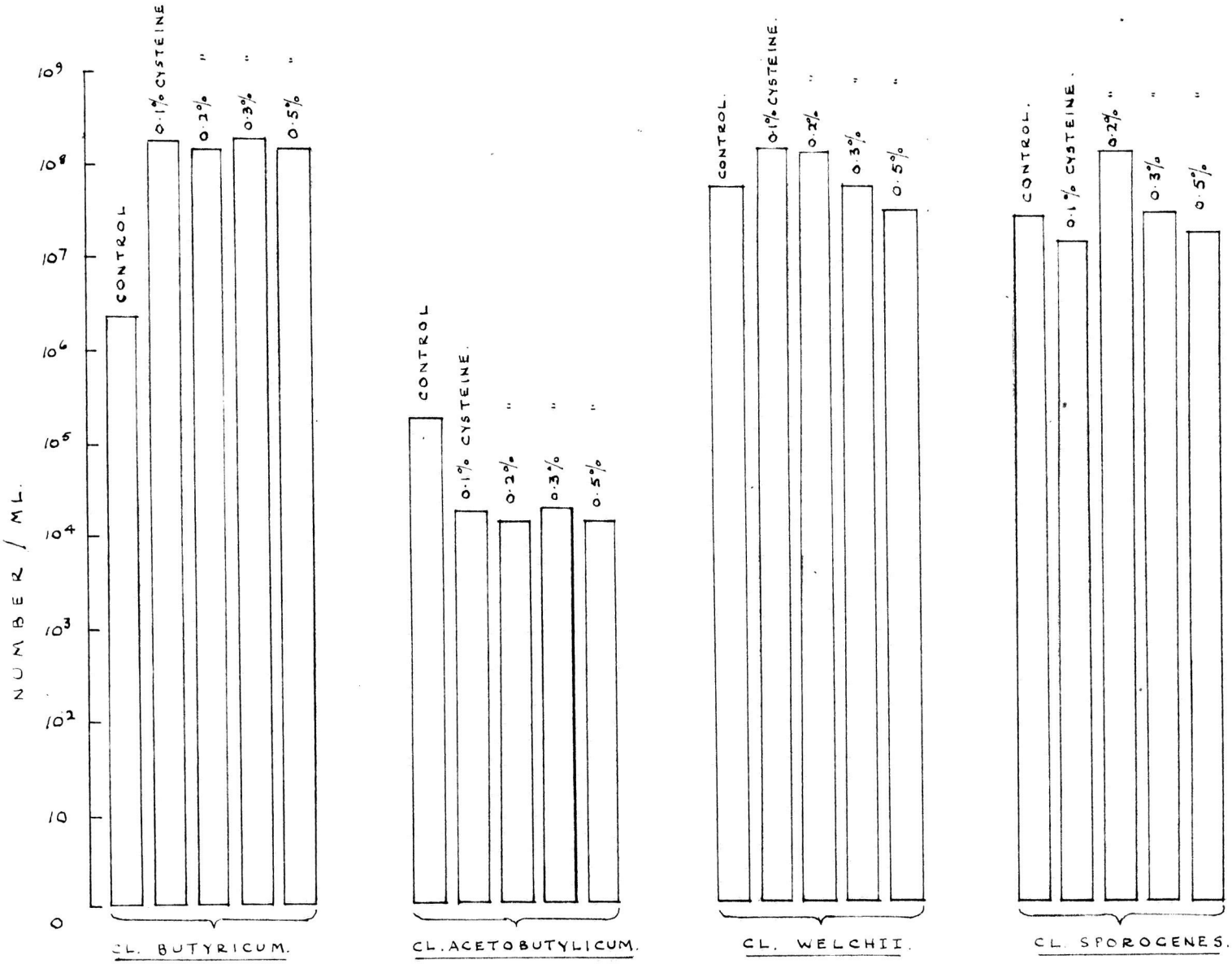


FIG 4. EFFECT OF SODIUM FORMALDEHYDE SULPHOXYLATE ON THE INITIATION OF GROWTH: RESULTS OF DILUTION COUNTS (3 REPLICATES) EXPRESSED AS MOST PROBABLE NUMBERS.

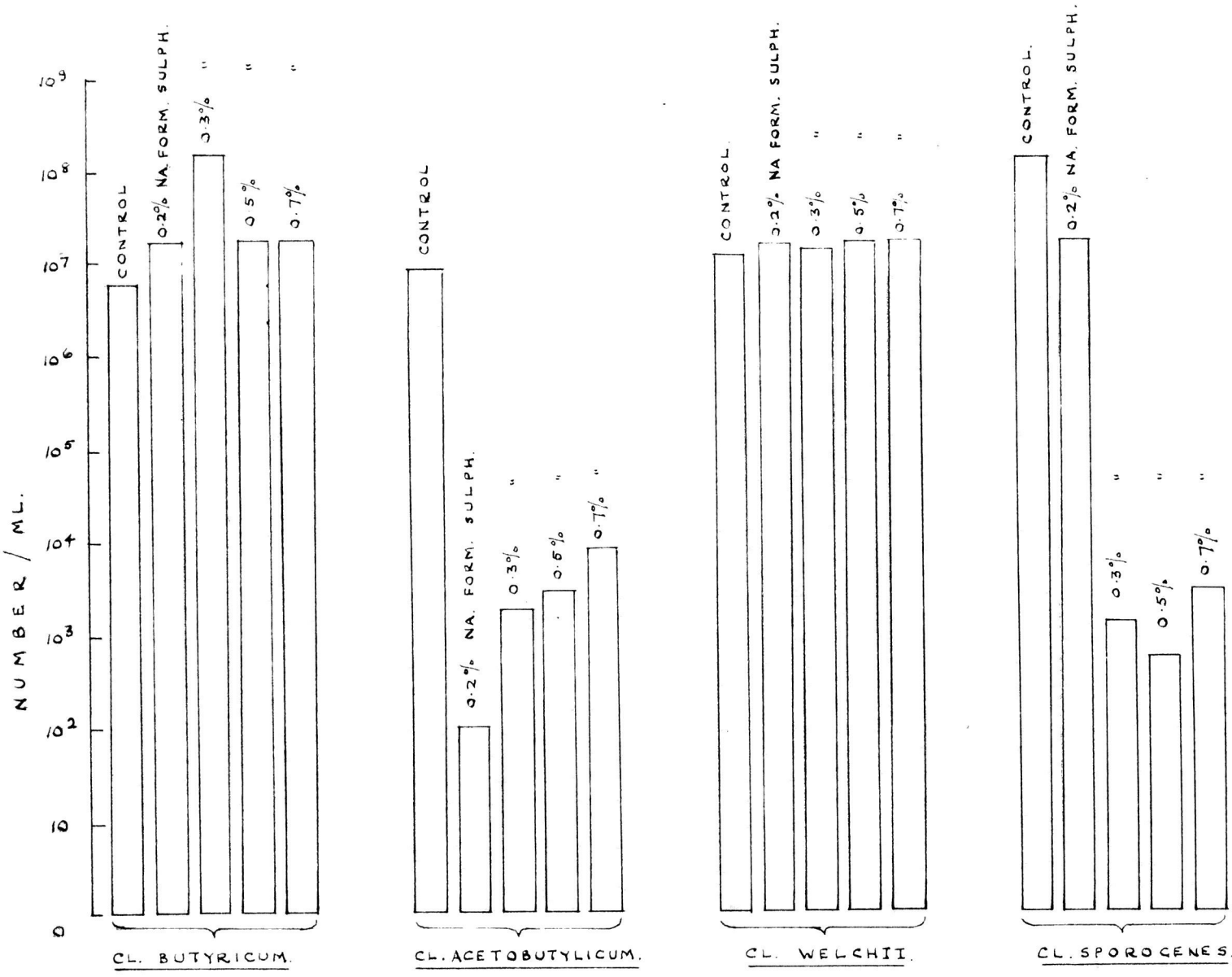
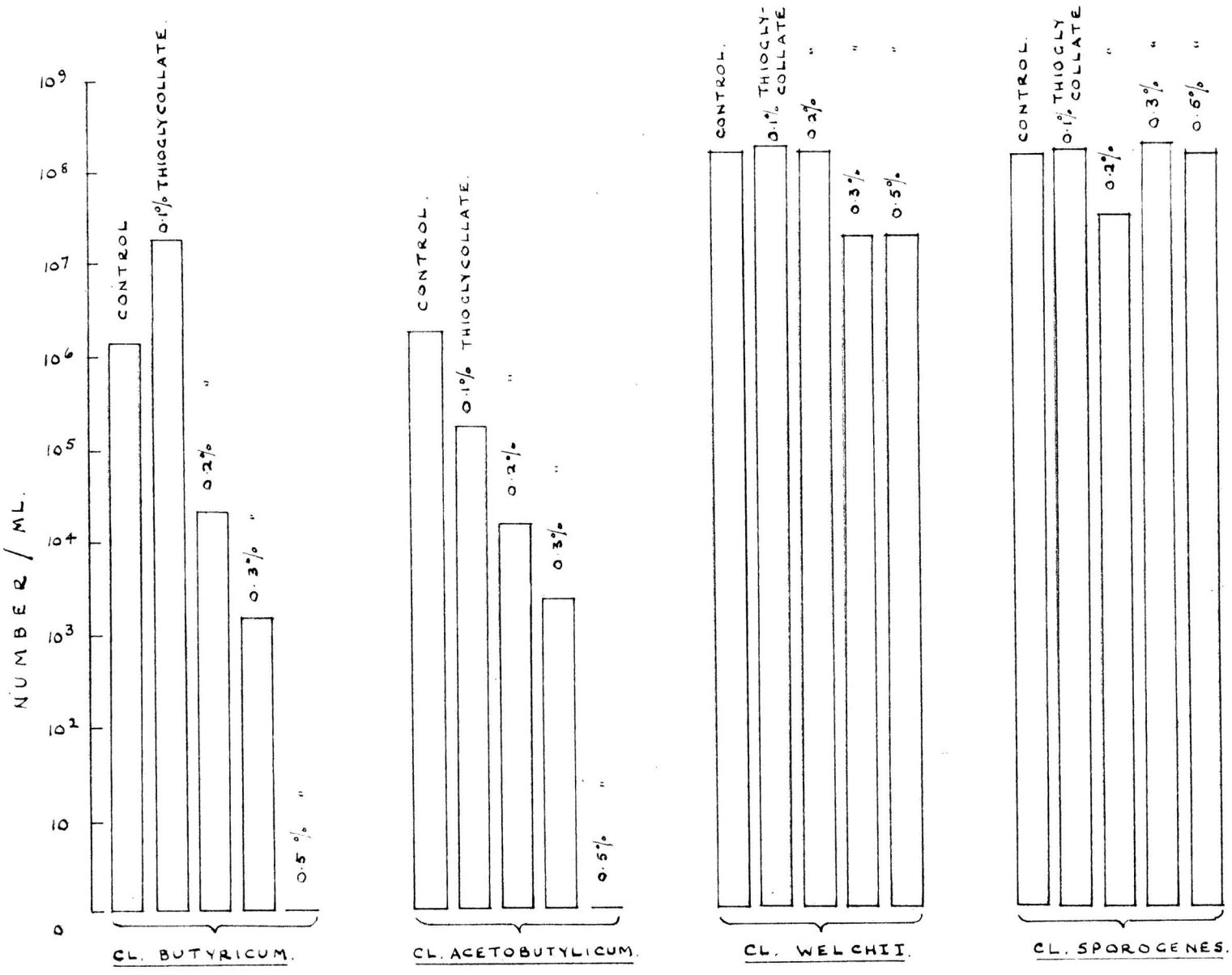


FIG 5. EFFECT OF THIOGLYCOLLIC ACID ON THE INITIATION OF GROWTH: RESULTS OF DILUTION COUNTS (3 REPLICATES) EXPRESSED AS MOST PROBABLE NUMBERS.



concentrations of thioglycollic acid or with the other reducing agents was compared. With the addition of 0.05% $\frac{v}{v}$ thioglycollic acid the semi-solid agar had been found, by a comparison with corn-liver mash and the coagulated blood medium of Pullar (1936), to be able to support growth from a small inocula of the four species. The evidence for this is presented in Table 3. The suitability of the blood medium for the cultivation of Cl. sporogenes is illustrated by data in Table 2. The results of the trials were interpreted by calculating the most probable numbers corresponding to the dilution end-points, using the tables provided by Hoskins (1934).

Of the three reducing agents, cysteine was the least inhibitory. When added in concentrations of up to 0.5% $\frac{w}{v}$ no adverse effect could be detected (Fig. 3). Sodium formaldehyde sulphoxylate and thioglycollic acid, each prevented the growth of small inocula of two of the species, unless added in low concentrations (Figs. 4 & 5). In all cases where marked depressions of counts were found, any growth which did occur was decidedly less luxuriant and slower in appearing than that obtained in the controls.

The toxicity shown by two of the three reducing agents varied with different species and it does not appear safe to generalise from these results about the action of the compounds on organisms other than the four investigated. The information obtained in the experiments described above does, however, allow an estimation to be made of (a) the amount of reducing agent required in semi-solid or solid media in order to ensure anaerobiosis over a specific period and (b) the type and concentration/

concentration of reducing agent which can be employed with success in the cultivation of certain types.

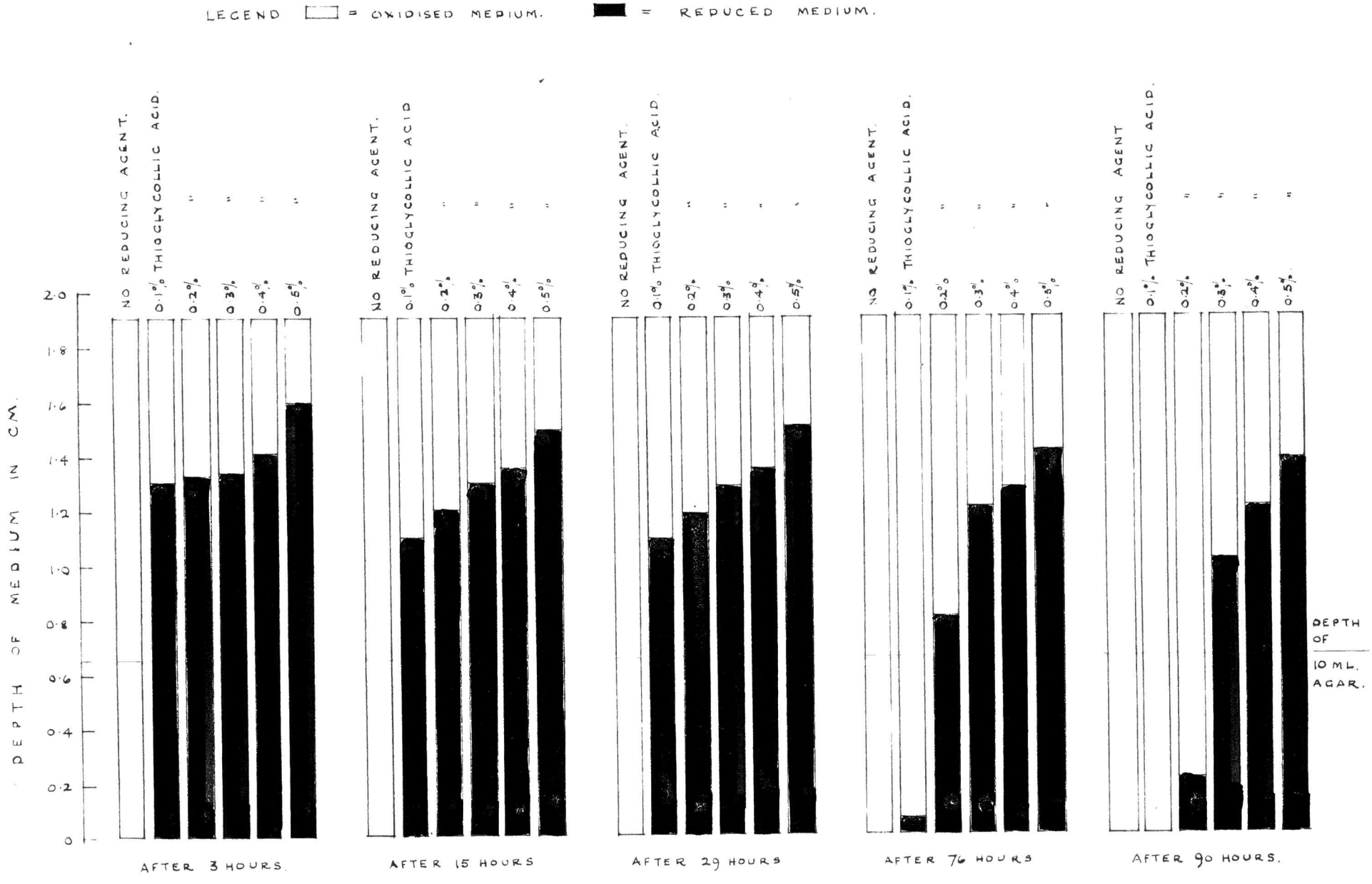
3. Methods for the isolation of pure cultures

The many methods for the isolation of pure cultures which have been described may be divided into two categories : (1) employing shake cultures in solid media and (2) employing surface cultures in Petri dishes or on agar slopes, special techniques being required to ensure absence of oxygen (McIntosh & Fildes, 1917; Burri, Staub & Hohl, 1919; Hall, 1929; Zeissler, 1929; Rosenthal, 1937; Brewer, 1942a). While all these methods are capable of yielding pure cultures, they also possess serious disadvantages.

In shake cultures many anaerobes form large amounts of gas and spread through the medium via the cracks produced by the gas. To remove colonies, a wire or capillary pipette has to be passed through a considerable depth of medium which may contain bacterial growth and the chances of contamination are fairly great. The operation has often to be repeated many times before pure cultures are obtained.

The preparation of surface cultures in Petri dishes or on slopes rendered anaerobic individually (e.g. McIntosh & Fildes, 1917; Cantor, 1941; Brewer, 1942a; Anderson, 1951) is time-consuming and requires special apparatus and with some techniques the colonies cannot readily be reached (Cantor, 1941; Anderson, 1951). The use of jars, in which several dishes or slopes may be incubated, makes the close observation of individual/

FIG 6. PENETRATION OF OXYGEN INTO 30 ML. OF AGAR IN 50 ML. CONICAL FLASKS.



individual cultures impossible.

The disadvantages of the methods discussed above could be overcome to a large extent if the medium were used in relatively shallow layers and without protection from oxygen. The possibility of achieving growth in shallow layers in 50 ml. conical flasks or Petri dishes, by the addition of reducing agents to the medium was therefore investigated.

The concentration of reducing agent which is required in order to keep agar/sufficiently reduced long to permit the initiation of growth by anaerobes, was determined by a technique similar to that described on p. 24 in connection with tests on the activity of reducing agents. 30 ml. of medium to which differing concentrations of thioglycollic acid had been added and which had been sterilised in screw-capped vials, were poured into Petri dishes or 50 ml. flasks and the progress of oxidation followed by measurements of the layer containing oxidised methylene blue. 30 ml. of agar filled 50 ml. conical flasks to a depth of approximately 1.9 cm. and Petri dishes to a depth of approximately 0.55 cm. Duplicate flasks or dishes were prepared in all cases and the average taken as the result.

Thioglycollic acid, in concentrations of 0.2% or higher, kept an 0.8 cm. or deeper layer of the agar completely reduced for 76 hours in the 50 ml. flasks (Fig. 6.). Tests showed that 50 ml. conical flasks are filled to a depth of 0.6 - 0.7 cm. by 10 ml. of agar. It therefore appeared possible to obtain growth of any except very slow growing species if (a) 10 ml. of medium is inoculated, poured into 50 ml. conical flasks and

after/

after solidification covered with 20 ml. of sterile agar and (b) the concentration of reducing agent employed is not toxic to the organism.

Attempts were made to cultivate strains of Cl. acetobutylicum lactate fermenter type 1 (see Description of pure cultures, p. 63), type 2 (p. 66), type 3 (p. 68) Cl. welchii, Cl. bifermentans and Cl. sporogenes by a technique such as is outlined above. In all, 25 strains were tested. A lactate agar (p. 23) was employed for the cultivation of types 1 - 3 and a peptone-glucose agar (p. 23) for the other species. 10 ml. of medium were inoculated from corn-liver mash cultures, poured into 50 ml. conical flasks and allowed to solidify. After solidification of the inoculated agar, 20 ml. of a covering agar (p. 23) were added.

All the strains grew well under these conditions. Growth from small inocula could be obtained regularly when loop dilutions, in the manner commonly used for the isolation of aerobic organisms from poured plates, were made. Disturbance of the medium through the evolution of gas was negligeable provided the number of colonies developing did not exceed 20 - 30. It may be mentioned here that during the search for suitable combinations of reducing agents, which was based on the data obtained from the tests described on p. 24 and p. 25 , the observation was made that the prescence of 0.05 or 0.1% w/v of sodium formaldehyde sulphoxylate and 0.2% w/v cysteine in the same medium made this medium unsuited for the growth of many strains of type 1. No other species was affected by this combination.

In/

FIG. 7. PENETRATION OF OXYGEN INTO 30 ML. AGAR IN PETRI DISHES.

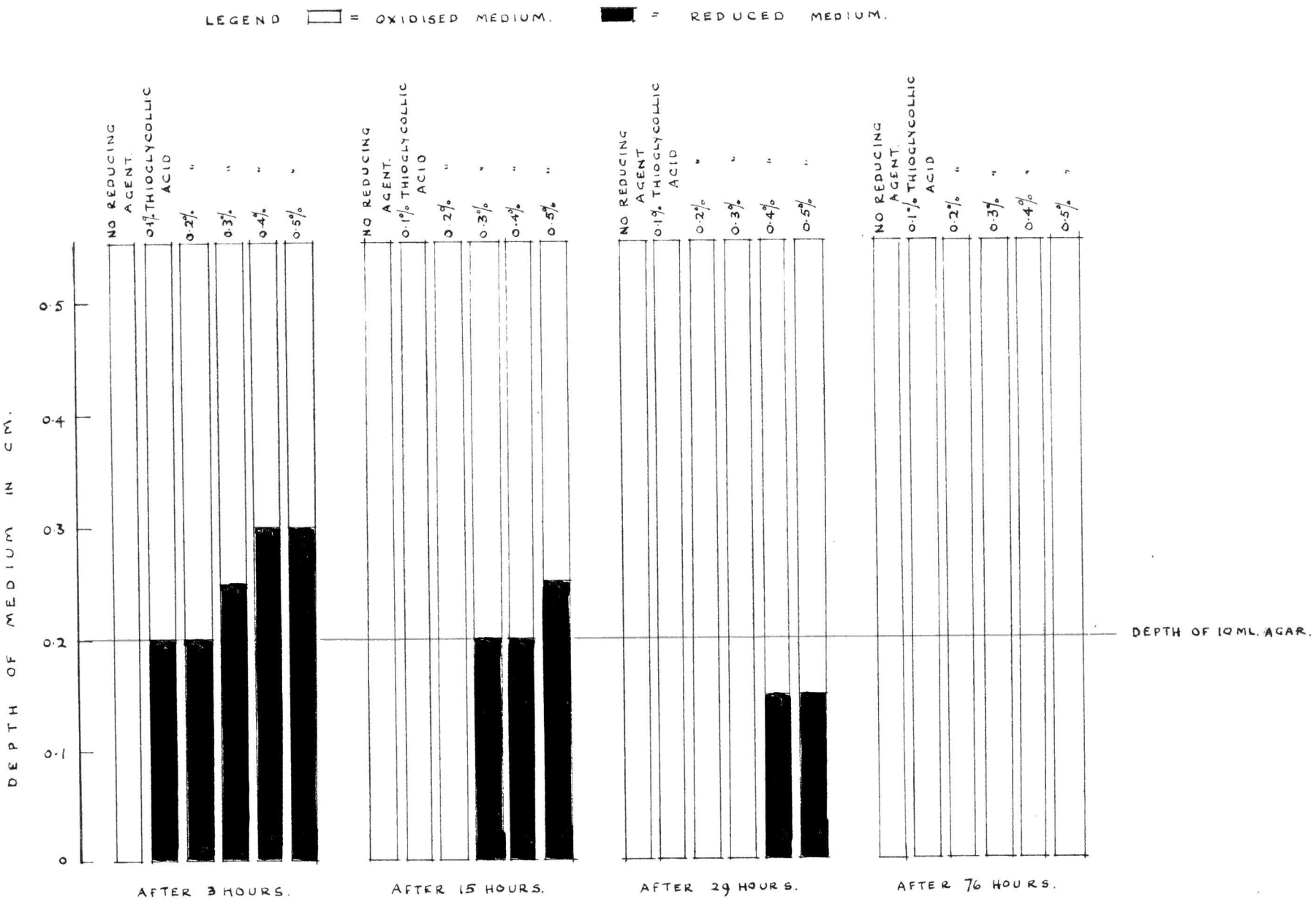


TABLE 4.

Growth of anaerobes in shake cultures in Petri dishes

Species	Medium	Dilution[1]	No. of Colonies
Cl. butyricum	Tomato agar	$1/10^4$	119
		$1/10^5$	38
		$1/10^6$	4
		$1/10^7$	nil
Cl. acetobutylicum	Tomato agar	$1/10^4$	30
		$1/10^5$	6
		$1/10^6$	nil
Cl. sporogenes	Liver extract-casein agar	$1/10^5$	many
		$1/10^6$	many
		$1/10^7$	26

1. Decimal dilutions of corn-liver mash cultures prepared in water.

In Petri dishes, concentrations of thioglycollic acid greater than 0.3% were required to keep a layer of 0.15 cm. depth completely reduced for 30 hours and with the highest concentration tested (0.5%) oxidation was complete at 76 hours (Fig. 7). 10 ml. of agar were found to fill Petri dishes to a depth of approximately 0.2 cm., and it appeared possible that species capable of rapid growth might be cultivated successfully in dishes using a technique similar to the one employed for flasks. Due to the shallower layer of agar and the greater accessability of the medium Petri dishes possess several advantages over flasks for the purpose of isolating pure cultures.

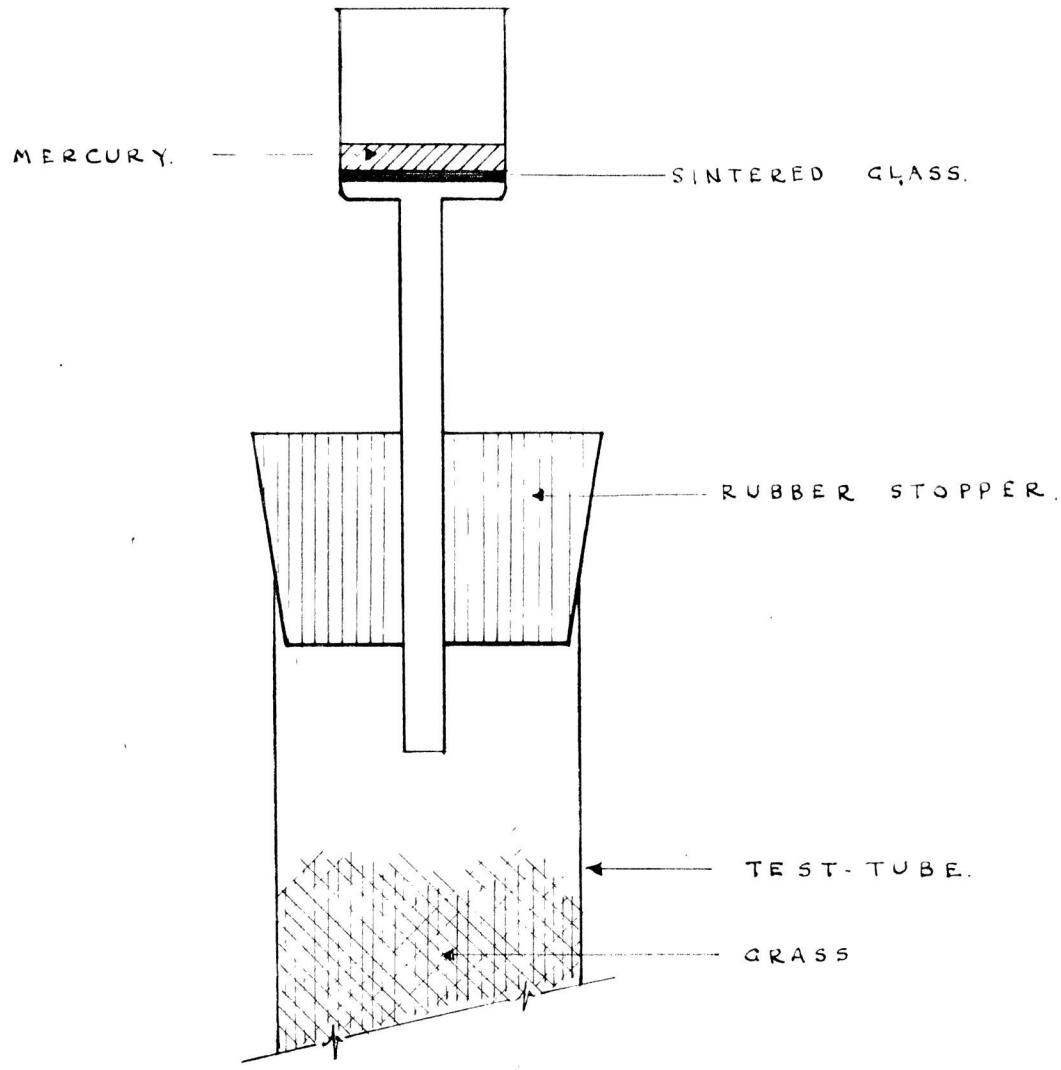
Further trials were conducted with pure cultures and tomato or liver extract-casein agar (p.23). 10 ml. of medium were inoculated from decimal dilutions of corn-liver mash cultures, poured into Petri dishes and after solidifcation 20 ml. of water agar containing 0.5% ≯ thioglycollic acid were added.

The results achieved were surprisingly good (Table 4), particularly when the toxicity exhibited by thioglycollic acid towards Cl. butyricum and Cl. acetobutylicum in tubed media is considered (Fig. 5, p.26). The proportion of the thioglycollate present in the oxidised form would be much greater in the Petri dishes than in the test-tubes and the explanation of the good growth obtained with Cl. butyricum and Cl. acetobutylicum may lie in this fact.

Shake cultures in 50 ml. conical flasks or Petri dishes have/

have been employed with success for the isolation of pure cultures in the work described below. Since, however, the effect of reducing agents on species of anaerobes other than those tested appears to be unpredictable and the danger exists that some types might be suppressed by the relatively high concentrations of reducing substances needed, surface cultures and poured plates incubated in McIntosh & Fildes jars were also used throughout the investigation for the purpose of procuring pure cultures.

FIG. 8. ARRANGEMENT FOR THE PREPARATION OF SILAGE IN TEST-TUBES.



Isolation of the dominant species of anaerobes in ensiled grass and discussion of the results

The materials from which isolations were attempted were silages prepared in large test-tubes and grass ensiled under farm conditions in circular concrete silos.

Ensilage in test tubes was carried on along the following lines. Grass collected from plots sown with perennial ryegrass (commercial strain) was filled into 8 x 1¾ in. test-tubes, 50 g. of grass being packed into each tube. Compaction was achieved by applying pressure with glass or wooden rods having a diameter slightly less than that of the tubes. When filled, the tubes were closed with rubber stoppers fitted with mercury valves (Fig. 8). In this way the gas formed during the fermentation was permitted to escape while the entry of air was prevented. After closing with stoppers, the tubes were held at constant temperatures in thermo-statically controlled water baths.

In order to cover a variety of conditions, a proportion of the material was subjected to certain treatments before being packed into tubes, viz. cutting into 1 in. lengths, mincing or wilting, while in other instances the temperature of incubation was varied. Mincing was carried out by means of an ordinary household mincing machine, wilting by keeping the grass, spread on wire trays, for several hours at atmospheric temperature.

Series of tubes were filled with grass comparable in composition and the treatment received before ensiling, all tubes/

TABLE 5.

Dry matter content, crude protein content and treatment of grass employed in the preparation of test-tube and farm silages

Experiment	Dry matter content (%)	Crude protein content[1]	Treatment before ensilage	Temperature of incubation	Age of silages examined[3]
3 a	19.1	32.0	None	30°	0.25, 1,
b			Cut into 1 in. lengths	30°	1.5, 2, 4
c			Minced	30°	& 8
4 a	20.0	22.0	None	30°	0.5, 1, 3
c			Minced	30°	& 8
5 a	20.5	21.0	Cut into 1 in. lengths	40°	1.25, 5 & 9
b			" " " "	30°	
c			" " " "	22°	
6 a	19.0	20.4	Cut into 1 in. lengths	30°	2, 6 & 14
b			Wilted, cut into 1 in. lengths	30°	
A 1	2-	2-	Cut into 1 in. lengths	30°	110
2	2-	2-	" " " " + 2 g. peptone per 100g. grass	30°	140
Farm silages					
X	17.1[4]	17.9[4]	Bruised	-	140
Y	17.1[4]	17.9[4]	+ molasses	-	140

1 % of dry matter
2 not determined
3 in days
4 average values

tubes in one series being incubated at the same temperature. The examination at intervals of different tubes from such a series permitted the changes occurring during the fermentation to be followed without disturbance of anaerobic conditions.

The two farm silages which were examined were made from grass plus clover, using the aftermath of a first year ley, by Mr. Nash. One silo was filled after the addition to the grass of molasses at the rate of three gallons per ton of fresh material. In the other instance, the grass was ensiled after mechanical bruising by a "Silorator" and without the addition of molasses.

Details of the dry matter and crude protein contents of the grass (determined by Mr. Mabbit and Dr. Smith) are given in Table 5. The treatments of the grass prior to filling into test-tubes, the temperatures at which tubes were incubated and the age of silages examined are indicated in the same table.

The contents of the test-tube silos were prepared for bacteriological examination by removal, using a large sterile corkscrew, of all the material in one tube to the jar of a top-drive macerator. Sufficient sterile water was added to the silage (made from 50 g. of fresh grass) to bring the total weight to 300 g. and the silage plus water was macerated for two minutes. In the case of the farm-made silages, representative samples were taken from each layer as it was exposed during the feeding of the material to stock. These were bulked and a further sample of 50 g. taken and treated in the manner described above for the contents of the test-tubes.

The/

The procedure employed for the isolation of anaerobes involved the following steps: (a) The preparation of decimal dilutions from the liquid phase of the macerated silage, the technique of dilution differing from that described on p.19 only in that 1 ml. was added to 9 ml. of diluent. Water containing 0.5% ~~w~~ liver extract (Oxoid) was used as the diluent, (b) The transfer of 1 ml. portions of dilutions, ranging from the 3rd (in some cases the 1st) to the 9th, to tubes of corn-liver mash and coagulated blood, three tubes of both media being inoculated from each dilution. The ability of these media to support growth from small inocula for many species of anaerobes has already been discussed (p.26). Incubation was carried on at 37°, and (c) Any corn-liver mash culture showing gas production or the presence of large rodshaped or sporing bacteria and any culture in the blood medium which showed blackening or digestion was transferred to a broth in which abundant sporeformation by members of the genus Clostridium was expected.

This broth contained per litre of tap water 15 g. peptone (Evans), 1 g. glucose and chalk, the pH being adjusted to 7.2. The capability of such a medium to permit good sporulation was suggested by the literature on factors influencing sporeformation (Introduction, p. 9). In view of the work of Foster, Hardwick & Guirard (1950) on this subject, two modifications of the glucose-peptone-chalk medium were also employed, viz. (a) the addition of 10 g. of soluble starch per litre and (b) treatment with charcoal before sterilisation. The broth was incubated for 5 - 6 days at 37°C. in a McIntosh & Fildes jar.

(d)/

TABLE 6.

Identity, numbers and origin of pure cultures isolated from ensiled grass.

Experiment	Species and no. of strains isolated (in brackets)	Dilutions from which strains were isolated
3 a, b and c	Type 1 (3) & type 2 (8)	$1/10^2$, $1/10^4$, $1/10^5$, $1/10^6$, $1/10^7$
4 a and c	Type 1 (10), type 2 (4)	$1/10^5$, $1/10^6$, $1/10^7$
5 a and b	Type 2 (1), Cl. sporogenes (2) & Cl. bifermentans (1)	$1/10^3$, $1/10^4$
6 a and b	Type 1 (1) & Cl. welchii (2)	$1/10$, $1/10^4$
A 1 and 2	Cl. sporogenes (3)	$1/10^3$
Farm silages X and Y	Type 1 (1), type 2 (1) type 3 (3) & Cl. welchii (1)	$1/10$, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$

(d) Part of the broth cultures were pasteurized at 80°C for five minutes and the surviving organisms isolated by plating, in Petri dishes, on an agar containing per litre of tap water 10 g. peptone (Evans) 5 g. glucose, 15 g. agar and with the pH adjusted to 7.0. In some instances, where difficulties were raised by spreading colonies, the concentration of agar was increased to 4%.

Anaerobic conditions in the plates were ensured either by incubation in the McIntosh & Fildes jar or through the addition of 0.5% thioglycollic acid to the agar, the technique described on p. 30 for establishing conditions suitable to the growth of anaerobes in Petri dishes being followed.

From time to time modifications in the procedure outlined above were made. These involved (a) plating directly from the primary corn-liver mash or coagulated blood cultures on glucose-peptone agar, and (b) plating directly from the primary cultures on an agar containing 1% ≋ peptone (Evans), 1% Na lactate (70% syrup), 0.8% ≋ Na acetate, 1.5% ≋ agar and with the pH adjusted to 6.5 (Bhat & Barker, 1947).

In all, 41 pure cultures were obtained. The origin of the strains and their identity are described in full in Table 44 in the appendix and are summarised in Table 6. Descriptions of morphological and cultural characters and a discussion of the taxonomic position of types 1, 2 and 3 are given on p. 63 et sequitur.

The 41 strains could be divided into 2 groups on the grounds of ability to attack proteins or their breakdown products :

(a)/

(a) showing proteolytic activity viz. Cl. sporogenes, Cl. bifermentans and Cl. welchii and (b) showing no proteolytic activity viz. types 1, 2 and 3. All strains in the latter group fermented lactate when tested by the method outlined by Bhat & Barker (1947) i.e. in a medium containing, besides lactate, peptone and acetate.

Considering the many factors and their complex inter-relationships which must control the development of micro-organisms in ensiled grass and further the limited scope of the isolations described above, it is quite possible that some additional groups are of importance in silage. Yet, the finding that the dominant species of anaerobes were lactate-fermenting or proteolytic species is in agreement with results obtained by other workers. Species which have been shown to multiply in ensiled fodder are Cl. sporogenes, Cl. bifermentans and Cl. welchii, all of which possess at least some proteolytic powers, and saccharolytic organisms which are now considered to belong to the species known as Cl. butyricum (Introduction, p.2, 4; Breed, Murray & Hitchens, 1948). Some of these saccharolytic strains have been shown to ferment lactate while in others this ability has been disputed (e.g. van Beynum & Pette, 1935 - 36). When, however, the more recent investigations of Bhat & Barker (1947, 1948) are considered, it appears likely that failure to establish lactate attacking properties in such strains has been due to tests being carried out with pure cultures and without the addition of acetate to the medium.

There are also grounds, derived from purely theoretical considerations,/

considerations, for believing that anaerobes which can utilise lactate or proteins or their breakdown products will have good opportunities for flourishing in ensiled fodder. During ensilage an extensive micro-flora develops which metabolises carbohydrates and other carbon compounds present in the fodder. Considerable competition for such substances must exist. On the other hand, the ability to utilise lactates or proteins or their breakdown products under anaerobic conditions is, as far as is known, limited with occasional exceptions, to strictly anaerobic species (Stephenson, 1949), and such substances are present in considerable quantities in the great majority of silages (e.g. Watson, 1939).

All available evidence pointed to lactate-attacking and proteolytic species, including weakly proteolytic species such as Cl. welchii, as becoming dominant among the strictly anaerobic micro-organisms. Further efforts were therefore concentrated on devising selective media for such organisms.

The development of a selective medium for the enumeration of lactate fermenting anaerobes.

The most direct way of achieving selectivity for lactate attacking species would appear to be by the employment of a medium containing lactate as the sole source of energy.

This approach has been investigated by Dorner (1924), van Beynum & Pette (1935 - 36) and Hostettler, Sahli & Binz (1941a) and in no case has it proved successful. Lactate attacking species/

species never became dominant in such media, which indeed appeared to be selective for proteolytic anaerobes. The conclusions reached by the above authors were that media containing carbohydrates, in particular glucose, were essential for the successful primary cultivation of lactate fermenting anaerobes from silage and other materials.

New light has, however, been shed on the biochemistry of the butyric fermentation of lactate by the investigations of Bhat & Barker (1947; 1948). They showed that, when in pure or almost pure culture, organisms related to Cl. butyricum can ferment lactate only when acetate or pyruvate are also included in the medium. These findings, besides suggesting explanations for the failures mentioned above, indicated that a solution might after all be found along these lines. The growth of lactate fermenting anaerobes isolated from ensiled grass and of a laboratory stock culture of Cl. butyricum was therefore investigated on lactate media similar to those developed by Bhat & Barker (1947).

The media employed consisted of a basal medium to which, in different tests, were added varying concentrations of peptone (Evans) or yeast autolysate (prepared as described by Barker & Beck (1942)).

The basal medium contained, per litre of tap water, 14.3 ml. sodium lactate (70% syrup), 8 g. sodium acetate, 1 g. ammonium sulphate, 0.1 μg. biotin, 100 μg. para-amino-benzoic acid, a a mineral supplement (composition and method of addition described in the Appendix, p. 114), 0.5 ml. thioglycollic acid and/

and 2.5 g./agar. In tests where 1% ⅟ peptone was added, the mineral supplement was omitted.

Peptone and yeast autolysate were added before sterilization or, observing asceptic precautions, after the media had been tubed and autoclaved. In the latter case peptone was added from a 5% ⅟ aqueous solution of pH 6.0; the yeast autolysate was also adjusted to pH6 before sterilization. In all cases the pH of media was adjusted to 6.0 prior to autoclaving.

The media were dispensed in 4 - 5 ml. quantities in 5 x 0.5 in. test-tubes and sterilised at 22½ lbs. pressure momentarily in the autoclave. Prior to inoculation the tubes were heated in boiling water in order to melt the agar. If not used on the day of preparation, heating was extended to 10 minutes in order to drive off dissolved oxygen.

Inoculations were made either by loop from corn-liver mash cultures incubated for 24 to 48 hours at 37°C or by pipette, using 0.5 ml. quantities of decimal dilutions of corn-liver mash cultures prepared in sterile water. Growth was detected by visual inspection. For the purpose of comparing the quantities of insoluble gas produced during the fermentation, seals of water agar (containing 15 g. agar per litre of tap water) were poured on top of the inoculated semi-solid medium. To determine changes in the pH level of the medium, indicator dyes viz. bromo-thymol blue in concentrations of 0.0016% ⅟ or neutral red in concentrations of 0.0025% ⅟ were at first incorporated in the media. The dyes were, however, rapidly reduced during incubation and/

TABLE 7.

Growth and gas production by lactate fermenting anaerobes in lactate media (basal medium + 1% peptone).

Species	Culture	Growth		Gas production	
		Complete medium	Medium without lactate	Complete medium	Medium without lactate
Type 1	4-1	+++	+	4.8	0.4
	4-7	+++	+	5.6	0.2
	4-12	+++	+	5.5	0.4
	6-2a	+++	+	6.0	0.4
	6-2b	+++	+	6.0	0.4
	4-9	+++	+	6.0	+
	3-15	+++	+	6.0	+
	Cl. butyricum	+++	+	6.0	++
Type 2	3-5	+++	+	5.0	+
	3-6	+++	+	4.8	+
	3-12	+++	+	5.0	+
	3-13	+++	+	4.7	0.4
	3-16	+++	+	4.5	+
	4-4	+++	+	5.0	+
	31b	+++	+	4.4	++
Type 3	6-25	+++	+	5.5	+
	6-27	+++	+	5.2	+

Legend Growth: + - poor; ++ - fair; +++ - good

Gas production: + - small no. of bubbles; ++ - accumulation of bubbles; figures - rise of seal in cm.

TABLE 8.

pH changes produced by lactate fermenting anaerobes in lactate media (basal medium + 1% peptone)

Species	Culture	pH prior to inoculation (both media)	pH after 6d. at 37°C	
			Complete medium	Medium without lactate
Type 1	74	6.3	7.4	6.4
	Cl. butyricum	6.3	7.0	6.3
Type 2	3-6	6.3	6.9	6.4
	3-1b	6.3	6.8	6.4
	31b	6.3	7.0	6.4
Type 3	141	6.3	7.0	6.3
	145	6.3	7.6	6.4
	150	6.3	7.2	6.4

TABLE 9.

Growth, gas production and pH changes initiated by lactate fermenters in lactate media containing different concentrations of peptone. (After 5 days at 37°C)

Basal medium +

Species	Culture	0.05% peptone			0.1% peptone			0.25% peptone			0.5% peptone		
		Growth	Gas	pH	Growth	Gas	pH	Growth	Gas	pH	Growth	Gas	pH
Type 1	74	+++	2.7	7.2-7.4	+++	3.1	7.2-7.4	++++	2.6	7.4	++++	4.0	7.6
Type 2	3-16	+	+	6.3	+	0.6	6.5	++	1.5	6.8	+++	2.5	7.3
Type 3	150	+++	4.2	7.6	++++	4.2	7.6	++++	4.1	7.6	++++	4.7	7.6
Uninoculated	-	-	-	6.3	-	-	6.3	-	-	6.3	-	-	6.3

Growth: + - poor
++ - fair
+++ - good
++++ - very good

Gas production: + - small no. of bubbles;
figures - rise of seal in cm.

and the method finally employed was to withdraw part of the culture by means of a Pasteur pipette and to estimate the pH in a comparator.

It may be mentioned here that bromo-thymol blue in the concentration employed proved toxic to the majority of the type 1 strains tested.

In the basal medium plus 1% $\frac{w}{v}$ peptone all the strains tested grew well and produced considerable amounts of gas and a rise in the pH of the medium (Tables 7 & 8). The rise in pH is explained by the facts that butyric acid is a weaker acid than lactic and that more than one molecule of lactate must be attacked to permit the formation of one molecule of butyrate.

Since the presence of 1% peptone may afford opportunities for the growth of non-lactate fermenting species, the effect of reducing the amount of peptone added to the basal medium was investigated. Types 1 and 3 continued to grow well even with a concentration as low as 0.05%, but growth of type 2 deteriorated as the concentration of peptone was reduced and, with progressively poorer growth, gas production and pH change in the medium decreased (Table 9).

This was not the case, however, when yeast autolysate was substituted for peptone. The lowest concentration (1% $\frac{v}{v}$) of yeast autolysate tested permitted good growth of type 2 (Table 10) and also of types 1 and 3. Yeast autolysate, when prepared by the method described by Barker & Beck (1942), is stated by these authors to contain 0.5 to 1% nitrogen and 1% yeast/

TABLE 11.

Comparison of dilution counts in the basal lactate medium plus 1% yeast autolysate and in corn-liver mash

Species	Culture	Medium	End points of dilution counts[1]							M. P. N.[2]
			$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$	$1/10^7$	$1/10^8$	$1/10^9$	
Type 1	4-9	Lactate	+++	+++	+++	+++	+++	---	---	2.3×10^7
		Corn-liver mash	+++	+++	+++	+++	---	---	---	2.3×10^6
	Cl. butyricum	Lactate	+++	+++	---	---	---	---	---	2.3×10^4
		Corn-liver mash	+++	+++	+++	---	---	---	---	2.3×10^5
Type 2	3-12	Lactate	+++	+++	+++	+++	++-	---	---	9.3×10^6
		Corn-liver mash	+++	+++	+++	+++	+++	---	---	2.3×10^7
Type 3	6-25	Lactate	+++	+++	+++	++-	++-	+--	---	2.8×10^6
		Corn-liver mash	+++	+++	+++	+++	++-	+--	---	1.5×10^7

1 Three replicates inoculated from each dilution
2 Most probable number calculated from the tables of Hoskins (1934)

TABLE 12.

Comparison of dilution counts in the basal lactate medium plus different concentrations of yeast autolysate and peptone and in corn-liver mash.

Species	Dilution	End points of dilution counts[1]			
		+ 3% y. autolysate	+ 5% y. autolysate	+ 1% y. autolysate & 0.25% peptone	Corn-liver mash
Type 1 (Cl. butyricum)	$1/10^3$	+++	+++	+++	+++
	$1/10^4$	++-	+++	+++	+++
	$1/10^5$	+--	---	++-	---
	$1/10^6$	---	---	---	---
	$1/10^7$	---	---	---	---
	$1/10^8$	---	---	---	---
	[2] M.P.N.	1.5×10^4	2.3×10^4	9.3×10^4	2.3×10^4
Type 2 (3 - 6)	$1/10^3$	+++	+++		+++
	$1/10^4$	+++	+++		+++
	$1/10^5$	+++	+++		+++
	$1/10^6$	+++	+++		+++
	$1/10^7$	+++	+++		---
	$1/10^8$	---	+--		---
	$1/10^9$	---	---		---
	[2] M.P.N.	2.3×10^7	4.6×10^7		2.3×10^6

1 Three replicates inoculated from each dilution

2 Most probable number calculated from the tables of Hoskins (1934).

Cont. over/

Table 12. Cont./

Species	Dilution	End points of dilution counts[1]			
		+ 3% y. autolysate	+ 5% y. autolysate	+ 1% y. autolysate & 0.25% peptone	Corn-liver mash
Type 3 (6 - 25)	$1/10^3$	+++	+++	+++	+++
	$1/10^4$	+++	+++	+++	+++
	$1/10^5$	+++	+++	+++	+++
	$1/10^6$	+++	+++	+++	+++
	$1/10^7$	++-	+++	++-	+--
	$1/10^8$	---	++-	---	---
	$1/10^9$	---	---	---	---
	[2] M.P.N.	1.1×10^7	1.1×10^8	1.1×10^7	4.3×10^6

1 Three replicates inoculated from each dilution
2 Most probable number calculated from the tables of Hoskins (1934)

TABLE 10.

Growth, gas production and pH changes initiated by lactate fermenter type 2 (strain 3-16) in lactate media containing different concentrations of yeast autolysate. (After 4 days at 37°C)

	Yeast autolysate %					
	0	1	3	4	5	7
Growth	-	+++	+++	+++	+++	+++
Gas (cm. rise of seal)	-	3.1	2.5	3.9	4.2	4.5
pH	6.4	7.2	7.4	7.2	7.4	7.4

Growth: +++ - good

yeast autolysate would therefore be equivalent in nitrogen content to approximately 0.06% Evans peptone. The effect of yeast autolysate on type 2 strains is no doubt attributable to its high content of bacterial growth factors.

The next step taken was to determine if lactate media had the ability to support growth from small inocula of the three lactate fermenters. The tests carried out consisted of comparisons of the endpoints of dilution counts in corn-liver mash and the basal medium plus 1% v/v or more of yeast autolysate, and the results obtained indicate that growth from small inocula may indeed be achieved in lactate media, even when these contain low concentrations of yeast autolysate (Tables 11 and 12).

Lactate media containing acetate and small concentrations of yeast autolysate thus appear to offer good prospects of attaining the requirements sought. As they contain only low concentrations/

concentrations of organic compounds other than lactate or acetate, non-lactate fermenting species will have little opportunity of becoming dominant. Further, if complete specificity cannot be achieved along these lines and mixed cultures result when the media are inoculated from materials such as silage, gas production and rise in pH resulting from the butyric fermentation of lactate would appear to be suitable criteria for detecting the presence of lactate fermenters.

Species of facultative anaerobes, e.g. coli-aerogenes bacteria which can utilise lactate and acetate as energy sources in the presence of oxygen occur in silage. It is necessary, therefore, from the point of view of selectivity that all parts of the medium remain anaerobic during the whole of the incubation period. Tests with water agar, (p.22) using the reduction of methylene blue as a criterion of anaerobic conditions, showed that this could be conveniently achieved by the simple expedient of adding a reducing agent to the agar seal which serves in the measurement of gas production. Water agar tubed in 5 x 0.5 in. tubes and containing 0.1% ⅓ thioglycollic acid was oxidised to a depth of 1.25 cm. below the surface after standing for twelve days in contact with air, and under the same conditions water agar containing 0.1% ⅓ cysteine was oxidised to a depth of 1.75 cm. Since growth of types 1, 2 and 3 in lactate media has always been found to occur within 2 to 6 days, seals of 2.5 cm. depth and containing 0.1% of reducing agent will provide anaerobic conditions during the period of incubation plus a margin of safety. Reducing agents added to the medium itself will be

able/

TABLE 13.

Inhibition of a type 3 strain by 0.1% thioglycollic acid in seal (basal medium plus 3% y. autolysate and 0.05% cysteine).

Species	Dilution	End points of dilution counts[1]		
		Thioglycollate seal	Cysteine[2] seal	Corn-liver mash
Type 3 (150)	$1/10^4$	+++	+++	+++
	$1/10^5$	+++	+++	+++
	$1/10^6$	+++	+++	+++
	$1/10^7$	++-	+++	+++
	$1/10^8$	---	+++	+--
	$1/10^9$	---	++-	++-

[1] Three replicates inoculated from each dilution
[2] 0.1% cysteine

TABLE 14.

Comparison of dilution counts in the basal lactate medium plus 3% y. autolysate and 0.05% cysteine and sealed with water agar containing 0.05% thioglycollic acid plus 0.05% cysteine and in corn-liver mash.

Species	Medium	End points of dilution counts [1]						Most probable number[2]
		$1/10^4$	$1/10^5$	$1/10^6$	$1/10^7$	$1/10^8$	$1/10^9$	
Type 1 (74)	Lactate	+++	+++	+--	+--	---	---	7.5×10^5
	Corn-liver mash	+++	+++	+++	---	---	---	2.3×10^6
Type 2 (3-16)	Lactate	+++	+++	+++	+++	+--	+--	7.5×10^7
	Corn-liver mash	+++	+++	+++	+++	++-	---	9.3×10^7
Type 3 (216)	Lactate	+++	+++	+++	++-	+--	---	1.5×10^7
	Corn-liver mash	+++	+++	+++	+--	---	---	4.3×10^6
Type 3 (150)	Lactate	+++	+++	+++	+--	---	---	4.3×10^6
	Corn-liver mash	+++	+++	+++	+--	---	---	4.3×10^6

1 Three replicates inoculated from each dilution
2 Calculated from the tables of Hoskins (1934)

able to combine with the small amount of oxygen introduced during inoculation.

Tests of the ability of lactate media when sealed with agar containing reducing agents of types 1, 2 and 3 were carried out to support growth from small inocula, using the basal medium plus 3% ⅟ yeast autolysate, and 0.05% ⅟ cysteine. When the seals contained 0.1% ⅟ thioglycollic acid, partial inhibition of some strains of type 3 occurred (Table 13). However, on employing water agar containing 0.05% thioglycollic acid and 0.05% cysteine for the purpose of sealing, satisfactory results were obtained (Table 14).

In order to aid the interpretation of results from comparisons of dilution count endpoints in lactate media and in corn-liver mash, most probable numbers were calculated from the tables of Hoskins (1934) and the limits of error at a confidence interval of 1:3⅓ by the method of Fisher & Yates (1943). The latter were found to lie between 487 and 20.5% of the most probable number.

Using these limits, counts of a type 2 strain in the basal medium plus 1% ⅟ yeast autolysate were not significantly different from counts in corn-liver mash, and those of a type 3 strain were on the border line of being significantly less in the lactate medium (Table 11). In the same lactate medium one strain of type 1 gave a significantly higher result than in corn-liver mash, and a second strain a significantly lower result (Table 11). The numbers of cells in cultures of strains of types 1, 2 and 3 when estimated in the basal medium plus 3% yeast autolysate and 0.05% ⅟ cysteine, and in corn-liver mash (Table 14) showed no statistically significant differences. Among the data presented

in/

in Table 12 there is no instance where counts in the lactate media were significantly lower than in corn-liver mash.

Therefore, as corn-liver mash has been shown to be a medium suitable for the quantitative enumeration of organisms belonging to the Cl. butyricum group (McClung & McCoy, 1934; McClung, 1940), the conclusion was drawn that lactate media may also be relied upon to give accurate estimates of the numbers of such organisms.

The selectivity of the method was investigated by (a) tests conducted with pure cultures of obligate anaerobes, of species of the genus Bacillus, of coli-aerogenes bacteria and of species of the genera Streptococcus and Lactobacillus and (b) detailed examination of the microflora of tubes inoculated from dilutions prepared from ensiled grass (experiments 7 - 10, described on p.78 et sequitur).

The lactate media employed in the trials with pure cultures and in the examination of ensiled grass in the later stages of the work (experiments 7 - 10, p.78) embodied two slight changes in the basal medium from that described on p. . These changes were the reduction of the concentration of agar from 0.25 to 0.2% and the addition of 5 mg. of resazurin per litre of medium. A similar concentration of resazurin was also incorporated in the seals. The complete medium contained, besides the base, 3% yeast autolysate and 0.05% cysteine (i.e. total concentration of reducing agents 0.05% thioglycollic acid and 0.05% cysteine) and was adjusted to pH 6.0. The seals contained 0.5 ml. thioglycollic acid and 0.5 g. cysteine per/

TABLE 15.

Growth of pure cultures of facultative anaerobes in the selective lactate medium

Species	Lactate medium				Tomato-juice medium
	plus seal		no seal		
	Growth	Gas	Growth	Gas	Growth
Aerobic sporeformers					
B. licheniformis	-	-	S	-	
" "	-	-	S	-	
B. coagulans	-	-	S	-	
B. cereus	-	-	S	-	
B. polymyxa	-	-	S		
Coli-aerogenes bacteria					
D. 53	-	-	S	-	
D. 75	-	-	S	-	
D. 144	-	-	S	-	
D. 168	-	-	S	-	
D. 761	-	-	S	-	
Lactic acid bacteria					
Leuconostoc	-	-	-	-	+++
S. faecalis	+	-	+	-	+++
S. liquefaciens	-	-	+	-	+++
" "	+	-	+	-	+++
Heteroferm. Lactobacillus	-	-	-	-	+++
Homoferm. Lactobacillus	-	-	-	-	+++

Growth: + - poor +++ - good S - on surface only

per litre of water agar, the pH being adjusted to 7.0.

Gas production was measured as before by the rise of the agar seals. Changes in the pH level were measured by withdrawing part of the culture by means of a capillary pipette, mixing with one drop of bromo-thymol-blue on a spot plate and comparing the resultant colour with that obtained from an uninoculated tube.

Out of 94 pure cultures of anaerobes, isolated from ensiled grass during the course of this work, 25 showed good growth in the lactate medium when transferred by loop from corn-liver mash cultures. These 25 strains liberated hydrogen, raising the seal 3.0 cm. or more and altered the pH of the medium sufficiently to produce a blue colour with bromo-thymol-blue as compared to a yellowish green given by the control. All of these strains could be identified with type 1, 2 and 3.

The other 69 strains grew poorly to moderately in the selective medium, producing in the majority of cases either a few bubbles of gas or no gas. A small proportion of the cultures caused a rise of 0.2 - 0.4 cm. of the agar seal and strains of Cl. welchii a rise of 0.5 - 1.0 cm. Spot tests with bromo-thymol-blue showed no instance in which the pH of the medium had been raised sufficiently to turn the dye blue.

The results obtained with pure cultures of facultative anaerobes are presented in Table 15. Inocula were taken from young cultures on agar or, in the case of the lactic acid bacteria, from cultures in a semi-solid tomato-juice medium (1% w/v peptone (Evans), 1% w/v Lemco, 20% v/v tomato juice, 0.5% w/v glucose/

glucose, 0.075% w/v agar, pH 6.0.).

In order to minimise the carry-over of nutrients, material taken from the semi-solid medium was first diluted in water, and the viability of inocula of lactic acid bacteria was tested in the tomato-juice medium. Only S. liquefaciens and S.faecalis produced growth which became detectable by visual inspection and none of the cultures tested produced gas or altered the pH of the medium sufficiently to enable the change to be observed by the method employed. In several cases coli-aerogenes bacteria and aerobic sporeformers proliferated on the surface of the seal.

During the investigations of ensiled grass (p.78) in which lactate media were employed, observations on their selectivity as regards species of facultative anaerobes were continued by means of surface platings on glucose-peptone agar from many tubes. Aerobic sporeformers and coli-aerogenes bacteria were encountered very infrequently and then only when growth of such organisms had also occurred on the surface of the seal.

Gram positive rods and cocci, both types showing the properties of facultative anaerobes in glucose agar, were, on the other hand, isolated frequently. These organisms formed distinct colonies in the semi-solid medium, but never produced gas or a rise in the pH level. On continued transfers in the selective medium, growth became much poorer or did not occur at all and further growth appeared to be as good when lactate was omitted as in its presence.

The/

The selectivity of the method is thus not complete, and the appearance of growth cannot be used as an indication of the presence of lactate fermenting anaerobes. Approximate measurements of gas production and rise in the pH level of the medium do, however, appear to be reliable criteria for this purpose.

Further evidence regarding the reliability of these two criteria in the detection of the organisms under discussion was obtained by the investigation of tubes inoculated with dilutions prepared from ensiled grass and in which the presence of lactate attacking anaerobes was indicated. From 42 out of 44 tubes in which both tests showed that fermentation of lactate had occurred, pure cultures of types 1, 2 or 3 could be isolated by means of platings in petri dishes incubated in anaerobic jars. From the remaining two tubes pure cultures of lactate fermenter type 4 (p.70) were obtained. In two other cultures in which considerable evolution of gas but no rise of the pH level was detected multiplication of lactate fermenter type 5 (p.71) had taken place.

The evidence thus indicates that the method when applied to mixed populations of micro-organisms provides quantitative results. Since the technique is neither time-consuming nor space consuming and does not require elaborate apparatus, it appears to fulfil satisfactorily the initial aims.

The development of a selective medium for the enumeration of proteolytic anaerobes

Among the media which have been shown to allow growth from small inocula of Cl. sporogenes and related organisms and of Cl. welchii are meat-media containing proteins and their break-down products but little carbohydrate (McClung & McCoy, 1934; McClung, 1940). Where only proteins or related substances are present, it appears unlikely that any but proteolytic species will be capable of active growth and the possibilities inherent in such media were therefore investigated.

As complex meat media present difficulties in regard to preparation and achievement of anaerobic conditions in all parts of the medium and as the ingredients were not always readily available, attention was concentrated on semi-solid agar containing peptone. Trials were conducted with a medium containing per litre of tap water 15 g. peptone (Evans), 10 ml. yeast autolysate (Barker & Beck, 1942), 0.5 ml. thioglycollic acid, 2 g. agar and with the pH adjusted to 7.0 prior to sterilisation. The agar was tubed in 4 - 5 ml. quantities in 5 x $\frac{1}{2}$ in. test-tubes and sterilised in the autoclave.

To determine if quantitative growth of Cl. sporogenes, Cl. bifermentans, and Cl. welchii could be obtained in the semi-solid peptone agar, comparisons were made of end-points obtained from dilution counts with end-points in corn-liver mash. For this purpose corn-liver mash cultures were diluted in water and $\frac{1}{2}$ ml. portions of the dilutions inoculated into the media. The results of the

comparisons/

TABLE 16.

Comparison of dilution count and end-points in a semi-solid peptone medium and in corn-liver mash

Culture	Dilution count end-points[1]						
	$1/10^4$	$1/10^5$	$1/10^6$	$1/10^7$	$1/10^8$	$1/10^9$	M.P.N.[2]
Cl. welchii							
Agar	++++++	++++++	++++++	++++++	++----	------	4.8×10^7
Mash	++++++	++++++	++++++	++++++	+-----	------	4.1×10^7
Cl. sporogenes							
Agar	++++++	++++++	++++++	++++++	++++--	+-----	8.1×10^7
Mash	++++++	++++++	++++++	++++++	+++---	+-----	8.1×10^7
Cl. bifermentans							
Agar	++++++	++++++	++++++	+++++-	------	------	1.2×10^7
Mash	++++++	++++++	+++++-	------	------	------	1.2×10^6
Limits of error of M.P.N.[2] (Confidence interval of 1:3$3\frac{1}{3}$)	306% to	32.5%					

1 - dilutions made in water, 6 replicates inoculated
2 - most probable numbers and limits of error calculated from the tables of Fisher & Yates (1943)

comparisons are presented in Table 16, and indicate that the peptone medium is indeed suitable from the quantitative point of view.

To test specificity, inoculations were made by loop from young cultures of lactate-fermenting anaerobes, aerobic sporeformers, coli-aerogenes bacteria and lactic acid bacteria. As some of the species of facultative anaerobes involved have the ability to attack proteins and related compounds in the presence of oxygen, it was necessary to exclude this gas from all parts of the medium. This was achieved by sealing with an agar containing per litre of tap water 10 g. agar, 0.5 g. cysteine, 0.5 ml. thioglycollic acid, 5 mg. resazurin and with the pH adjusted to 7.0. Visible growth of several strains occurred, although this growth was far less luxuriant than growth in liquid peptone media containing glucose and incubated in anaerobic jars.

The position appeared to be similar to that reached with the selective lactate medium described in the previous section. While complete selectivity was not obtained, the growth of unwanted organisms seemed to be sufficiently hampered to prevent the suppression of proteolytic anaerobes, and if tests capable of detecting the anaerobes in mixed cultures could be devised, a satisfactory technique would be available.

In view of the fact that protein breakdown by facultative anaerobes in the absence of oxygen and fermentable carbohydrates is likely to be limited (Stephenson, 1949), the occurrence of proteolysis/

proteolysis was considered to be an indicator worthy of investigation. In all further tests, a modified form of the semi-solid peptone medium was employed and contained per litre of tap water 15 g. peptone (Evans), 10 ml. yeast autolysate, 120 g. gelatine, and 0.5 g. cysteine, the pH being adjusted to 7.0 prior to sterilization. Gelatine was introduced into the medium as its liquefaction is easily detected and is induced by strong and weakly proteolytic anaerobes alike. Cysteine was substituted for thioglycollic acid in order to provide a better opportunity for the detection of hydrogen sulphide producing bacteria, a purpose for which peptone alone is not entirely suitable (Tilley, 1923; Zo Bell & Feltham, 1934).

The gelatine medium was distributed in 4 - 5 ml. quantities in 5 x $\frac{1}{2}$ in. tubes and sterilised by autoclaving for 15 min. at 15 lbs. pressure, a treatment which did not affect its capacity to form a solid gel. In the trials, the medium was melted in a water bath at 50°C prior to inoculation and the inoculated medium was solidified by placing in cold water. When solid, seals of water agar (composition as stated on p.44) were poured. In the case of obligate anaerobes and lactic acid bacteria, inocula were taken from corn-liver mash cultures incubated for 24 - 48 hours at 37°C. The mash cultures were diluted in water to minimise the carry-over of nutrients, and were transferred by loop. Coli-aerogenes bacteria and aerobic sporeformers were cultivated in peptone broth and inoculations made by loop. All cultures were incubated at 37°C.

Five/

TABLE 17.

Growth and proteolysis in the selective protein medium by pure cultures of proteolytic and weakly proteolytic anaerobes.

Species		Growth	Gas	NH_3	H_2S	Indole reagents	Gelatin liquefaction
Cl. sporogenes	31a	+++	+	+++	+++	+ (violet)	+
	31c	+++	++	+++	+++	+ (")	+
	616	+++	1.2	+++	+++	+ (")	+
	635	+++	3.2	+++	+++	+ (")	+
	670	+++	0.3	+++	+++	+ (")	+
Cl. bifermentans							
	30	+++	+	+++	+++	+ (red)	+
	401	+++	+	+++	+++	+ (")	+
	342	+++	++	+++	+++	+ (")	+
	322	+++	+	+++	+++	+ (")	+
Cl. welchii	6-3	++	1.2	+	+	-	+
	304	++	1.8	+	+	-	+
	305	++	1.3	+	+	-	+

Growth: ++ - good
+++ - very good

Gas: + - occasional bubbles
++ - accumulation of bubbles
figures - rise of seal in cm.

NH_3: + - weak reaction
H_2S +++ - very strong reaction

Indole reagent: + (violet) - skatole or related compounds
+ (red) - indole

Five tests were used to determine if an attack on the constituents of the medium had taken place. These involved (a) a rough measurement of gas production by the appearance of gas bubbles or a rise of the agar seal; tests for (b) ammonia and (c) hydrogen sulphide, carried out by withdrawing part of the culture with a Pasteur pipette and testing on a spot-plate, in the former case with 1 drop of Nessler's reagent and in the latter with 1 drop of a saturated aqueous solution of lead acetate; (d) testing for indole and related substances by means of a spot-plate and the technique described by Roessler & McClung (1943); and (e) determination of gelatin liquefaction by placing the cultures in cold water. These tests all have the common feature of being neither time-consuming nor presenting special difficulties.

The results obtained with pure cultures of proteolytic anaerobes are presented in Table 17. Strains of Cl. sporogenes and Cl. bifermentans proved to be variable in the amount of gas liberated, but gave evidence of proteolysis with the other tests. With the indole reagents, the violet colour described by Spray (1936) was obtained from cultures of Cl. sporogenes. Spray (1936) considered this reaction to be of diagnostic value in mixed cultures. Strains identified as Cl. welchii produced gas and liquefied the gelatin, but the cultures gave only weak reactions when tested for ammonia and hydrogen sulphide.

Among the lactate fermenters, types 1 and 2 initiated growth and liberated gas but showed no signs of proteolytic activity./

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TABLE 18.

Growth and proteolysis in the selective protein medium by pure cultures of saccharolytic anaerobes.

Species	Growth	Gas	NH_3 production	H_2S production	Indole reagents	Gelatin liquefaction
Type 1 3-15	+	1.2	-	-	-	-
275	+	0.9	-	-	-	-
311	+	1.5	-	-	-	-
Type 2 3-12	+	+	∓	∓	-	-
3-16	+	0.4	-	-	-	-
338	+	0.5	∓	-	-	-
Type 3 6-25	-	-	-	-	-	-
145	-	-	-	-	-	-

Growth: + - poor
Gas: + - occasional bubbles
figures - rise of seal in cm.

NH_3 & H_2S: ∓ - doubtful reaction

TABLE 19.

Growth and proteolysis in the selective protein medium by pure cultures of coli-aerogenes bacteria and aerobic sporeformers

Species	Growth	Gas	H_2S production	NH_3 production	Indole reagents	Gelatin liquefaction
Coli-aerogenes 589	+	-	+	+	+ (red)	-
bacteria 573	+	+	+	+	+ "	-
403	+	-	+	+	+ "	-
466	+	+	+	+	-	-
427	+	-	+	∓	-	-
545	+	-	+	∓	-	-
168	+	+	+	+	-	- *
Aerobic sporeformers						
B. carotarum	+	-	+	+	+ (red)	-
B. pumilis	+	-	-	+	-	-
B. brevis	∓	-	-	-	-	-
B. polymyxa	+	+	-	-	-	-
B. mycoides	+	-	-	+	-	-
B. licheniformis	+	-	∓	+	-	-
B. circulans	+	-	-	+	-	-
B. cereus	+	-	+	+	+ (red)	-

Growth: ∓ - doubtful
+ - poor
Gas: + - occasional bubbles.

NH_3 & H_2S: ∓ - doubtful
+ - weak reaction

Indole reagents: +(red) - indole or related substances.

* this strain liquefies gelatine under aerobic conditions.

TABLE 20.

Growth and proteolysis in the selective protein medium by pure cultures of lactic acid bacteria

Species	Growth	Gas	NH_3 production	H_2S production	Indole reagents	Gelatin liquefaction
S. faecalis	+ ++	-	-	-	-	-
S. liquefaciens	++	-	-	-	-	+
S. liquefaciens	++	-	-	-	-	+
Leuconostoc	±	-	-	-	-	-
Homoferm. Lactobacillus	±	-	-	-	-	-
Heteroferm. Lactobacillus	-	-	-	-	-	-

Growth: ± - doubtful
+ - poor
++ - good

activity. Type 3 strains did not find conditions suitable for growth (Table 18).

The selectivity of the medium against coli-aerogenes bacteria and aerobic sporeformers is well demonstrated by the fact that species normally capable of liquefying gelatine did not do so (Table 19). Some strains did, however, show sufficient activity to produce substances reacting with the indole reagents and to give weak positive reactions in tests for ammonia and hydrogen sulphide. Among the lactic acid bacteria S. liquefaciens was found to grow well and to liquefy the gelatine, although ammonia and hydrogen sulphide could not be detected in the cultures (Table 20).

The strongly proteolytic anaerobes thus distinguish themselves in the selective medium by liquefaction of the gelatine and profuse production of ammonia and hydrogen sulphide. Cl. welchii cannot be recognised with certainty by its attack on the gelatine as S. liquefaciens produces the same reaction. In tubes where gelatine liquefaction but no other indications of proteolysis are found, isolations would be necessary to determine the presence or absence of Cl. welchii.

Trials with pure cultures of obligate and facultative anaerobes were continued throughout the course of the work as fresh strains were isolated. 31 cultures identified as Cl. sporogenes, 15 as Cl. bifermentans, 6 as Cl. welchii and 25 cultures comprising lactate fermenters types 1, 2 and 3 were tested in the selective medium, using the methods previously described. All strains produced reactions similar to those noted for these species in Tables 17 and 18. Cl. bifermentans was/

was further found to sporulate actively in the medium, producing the characteristic sporangium described by Breed, Murray & Hitchins (1948).

Obligate anaerobes which differed in character from the species mentioned above were on occasion obtained from ensiled grass. None of these strains liquefied gelatin and they comprised, as judged by morphological and cultural tests (see Description of species, p. 75) several distinct types. Two types showed proteolytic activity when inoculated into the gelatin medium viz. type 6 which liberated ammonia, hydrogen sulphide and indole and type 7 which liberated ammonia, hydrogen sulphide and skatole or a related compound.

The facultative anaerobes employed in this latter stage of the trials comprised 56 strains identified as belonging to the coli-aerogenes group of bacteria, 40 strains of gram positive cocci producing acid but no gas from glucose, including 6 strains capable of liquefying gelatin, and 4 strains of gram positive rods whose characters indicated a relationship to members of the genus Lactobacillus. The results obtained with these cultures were similar to the reactions listed in Tables 19 and 20 for coli-aerogenes and lactic acid bacteria.

Investigations of the value of the selective medium were also made by detailed examinations of the micro-flora of tubes inoculated from dilutions of ensiled grass in experiments 7 - 10 (p. 78). 38 tubes, in which the tests described above showed strong reactions for ammonia and hydrogen sulphide together with/

with liquefaction of the gelatin, all yielded pure cultures of either Cl. sporogenes or Cl. bifermentans. Further, the presence of Cl. sporogenes could be predicted by the characteristic reaction with the indole reagents and that of Cl. bifermentans from the presence of characteristic sporangia.

In twelve cases where gelatin liquefaction but no other indications of proteolysis were found, 6 of the tubes yielded pure cultures of Cl. welchii and the other 6 cultures of gram positive cocci capable of liquefying gelatin. No instance was found in which gelatin liquefaction appeared to be the result of action by aerobic sporeformers or gram negative rods. From 90 tubes, in which no attack on the gelatin and weak positive reactions in tests for ammonia and hydrogen sulphide were detected, pure cultures of coli-aerogenes bacteria, gram positive facultatively anaerobic cocci, gram positive facultatively anaerobic rods, lactate fermenting anaerobes attacking neither lactate nor gelatin were isolated. The contents of many of these latter tubes gave positive reactions with the indole reagents.

A solution to the problem of enumerating vegetative cells and spores of Cl. sporogenes and Cl. bifermentans appears to be provided by the selective medium. Differentiation of the two species without recourse to isolations is made possible by the reaction of cultures of Cl. sporogenes with the indole reagents and the appearance of the sporulating cells of Cl. bifermentans. In the case of Cl. welchii, the situation

is/

is less satisfactory, as isolations are required. Further, through the capability of saccharolytic anaerobes to initiate growth in the gelatin medium, an opportunity is provided for the isolation, by a non-selective method, of such species. The possible danger of relying exclusively on selective media is thus avoided.

E X P E R I M E N T A L

Part II.

INVESTIGATIONS OF THE GROWTH OF ANAEROBES IN ENSILED GRASS AND THE FACTORS CONTROLLING THEIR DEVELOPMENT.

Objects

The following pages contain an account of the application of the selective methods evolved to the examination of ensiled grass. In the experiments, information was sought about the identity of the species which proliferated and the extent of their multiplication, the stage of the fermentation at which multiplication takes place, the effect of varying certain factors, and the correlation of bacteriological with chemical results.

TABLE 21.

Treatments employed to investigate the effects of specific factors

Treatment	Designation in future tables
Cut into 1 in. lengths, incubated at 30° C.	30° or Standard
Cut into 1 in. lengths, incubated at 40° C.	40°
Cut into 1 in. lengths, incubated at 22° C.	22°
Uncut, incubated at 30° C.	Uncut
Minced, incubated at 30° C.	Minced
Cut into 1 in. lengths, sterile water added, incubated at 30° C.	+ water
Wilted, cut into 1 in. lengths, incubated at 30° C.	Wilted
Wilted, sterile water added, cut into 1 in. lengths, incubated at 30° C.	Wilted + water
Cut into 1 in. lengths, Lactobacillus culture added, incubated at 30° C.	+ lactobacilli

Methods employed in the preparation of the silages and their examination

The major part of the work has been conducted with grass ensiled in large test-tubes. Besides this, the selective methods were used on a small number of samples taken from the farm silages described on p. 33

Grass was ensiled in test-tubes by the method indicated on p. 32 . This method permits silage to be prepared under controlled conditions and the changes occurring during the fermentation to be followed. The material was taken from plots sown with pure strains of perennial ryegrass (S. 23 or S. 24). S. 24 was used for experiments 7 - 9 and S. 23 for experiment 10. To investigate the effects of variation in temperature, degree of chopping, moisture content and inoculation with lactobacilli on the ensilage process, the grass was ensiled under nine, slightly differing, conditions in each experiment. Details of the various treatments are given in Table 21.

Cutting into 1 inch lengths was carried out by means of sterilised scissors, mincing with a sterilised household mincing machine. Wilting was effected by passing a current of warm air from a hair dryer through grass spread on wire trays. Lactobacilli cultures used for inoculation were prepared in the laboratory by Mr. Keddie. Details of the farms silages have already been given (p. 33 and Table 5).

The preparation of the silages for bacteriological examination was as stated on p. 33 . Dilutions of the macerated material/

material were made in water by adding 1 ml. to 9 ml. of diluent. 0.5 ml. of the dilutions were transferred to the two selective media (composition and method of achieving anaerobiosis as stated on p.44 and p.50), three replicates being inoculated from each dilution. Incubation of cultures was carried on at 37°.

End-points of the dilution counts were obtained by observing gas production and change in pH level in the case of the lactate medium (p.39) and NH_3, H_2S and indole production gelatin liquefaction in the case of the and/gelatin medium (p. 51). In a proportion of tubes these presumptive counts were confirmed by isolations in 50 ml. flasks (p.28) or in Petri dishes incubated in the McIntosh & Fildes jar. The media employed for this purpose were glucose-peptone-liver extract agar (p.23), lactate-acetate-yeast autolysate agar (p.23) and tomato juice-peptone agar (p.23). To aid in the interpretation of results, most probable numbers were calculated from the dilution endpoints using the tables provided by Hoskins (1934). The error of the most probable number at a confidence interval of 1:33⅓ was derived according to the method of Fisher & Yates (1943), using the variance of the logarithm of the most probable number. The limits of error lie between 487 and 20.5%. Most probable numbers will be given as organisms per g. dry matter of the fresh grass employed. Dilutions, when quoted in succeeding parts of the text and tables, represent dilutions of the wet silage.

Information on the species of anaerobes proliferating was obtained in two ways: (a) by isolation and study of morphological/

morphological and cultural characters, and (b) tests conducted on mixed cultures. When dealing with tubes of lactate medium, these latter tests consisted of transferring part of the primary cultures to corn-liver mash and examining the mash cultures for the presence of cells storing iodophilic compounds. The occurrence of such cells was taken to indicate that anaerobes of type 1 were present in the primary tubes. Primary cultures in the gelatin medium which showed evidence of proteolysis were taken to contain Cl. sporogenes if a violet colour was obtained with the indole reagents (p. 51), and Cl. bifermentans if microscopical examination revealed the presence of characteristic sporangia.

Numbers of lactobacilli were determined by Mr. Keddie, using a selective medium (Keddie, 1952). Chemical analyses were carried out by Mr. Mabbit. For the qualitative detection of volatile acids partition chromatography was employed, (Hiscox & Berridge, 1950) quantitative determinations of butyric and acetic acids were made according to the method of Wiegner.
Lactic acid was determined as described by Barnett (1951). Standard Conway micro-diffusion units were used in the estimation of volatile base.

Description of pure cultures isolated

Media and methods employed

In the media described below, unless otherwise stated, the brand of peptone employed was Evans, and of liver extract and dessicated liver Oxoid. Yeast autolysate was prepared as described/

described by Barker & Beck (1942). Unless otherwise stated, all media were sterilised in the autoclave at 22½ lbs. pressure. Incubation of cultures apart from determinations of optimum and limiting temperatures was carried on at 37°.

Morphology. (1) Corn-liver mash: prepared as described by McClung & McCoy (1934), but with 1.6% w/v dessicated liver and 0.4% w/v liver extract instead of 1-2% finely ground dried liver.

(2) Liver extract-peptone semi-solid agar: 10g. liver extract, 5g. peptone, 5g. Bacto-tryptone, 1g. glucose, 1g. cysteine HCl, 0.5 ml. thioglycollic acid and 2g. agar per litre of tap water and with the pH adjusted to 7.0.

(3) Liver extract peptone broth: as 2 but without the addition of agar.

(4) Peptone-glucose semi-solid agar: 10g. peptone, 5g. liver extract, 10 ml. yeast autolysate, 10g. glucose, 0.5g. cysteine HCl, 0.5 ml. thioglycollic acid and 2g. agar per litre of tap water and with the pH adjusted to 7.0.

(5) Horse-flesh digest agar: The digest was prepared as described by Mackie & McCartney (1945, p. 102). To the digest were added 1% w/v peptone and 1% w/v agar and the pH adjusted to 7.0.

The size and shape of cells were determined by the examination of heat-fixed films of cultures grown in medium 1. The films were stained by Gram's method. Motility was investigated by microscopical examination of young cultures in 1 and 3. Further data on motility was obtained through the preparation of stab cultures in 2 and subsequent observation as/

as to whether growth spread away from the stab or not. Wet mounts of cultures in 1 and 4 were examined to determine ability to store iodophilic compounds. Information on the size, shape and position of spores was obtained from stained films of stab cultures in 5 or cultures in the selective lactate medium (p.44). The Schaeffer-Fulton modification of the Wirtz spore stain (Schaeffer & Fulton, 1933) and simple staining with dilute fuchsin were employed.

Cultural characteristics. (6) Liver extract-glucose agar: 10g. liver extract, 5g. peptone, 5g. glucose, 0.5 ml. thioglycollic acid, and 15g. agar per litre of tap water and with the pH adjusted to 7.0.

(7) Coagulated blood medium: prepared as described by Pullar (1936).

(8) Iron-glucose-gelatin: 120g. gelatin (Cox), 1g. glucose, 10g. peptone per litre of tap water and with the pH adjusted to 7.0. An iron nail was added to each tube and the medium autoclaved for 15 minutes at 15 lbs. pressure.

(9) Iron-litmus-milk: Litmus-milk was prepared as described by Cunningham (1947). Prior to sterilisation by intermittent steaming an iron nail was added to each tube.

(10) Tryptone-peptone semi-solid agar: 10g. Bacto tryptone, 10g. peptone, 0.5g. glucose, 0.5 ml. thioglycollic acid and 2.0g. agar were added per litre of tap water and the pH adjusted to 7.0.

(11) Fermentation medium: 10g. peptone, 30 ml. yeast autolysate, 0.05g. cysteine HCl and 2g. agar were added per litre of tap/

tap water. 1% of the substance under test was further added and the pH adjusted to 7.0. In the case of starch 0.2% was incorporated in the medium. In some instances, test substances were sterilised in 10% aqueous solution and added with aseptic precautions to the tubed and sterilised fermentation medium. Adjustments to the pH level were made when necessary to produce a final value of 7.0.

(12) Covering agar: 0.5g. cysteine HCl, 0.5 ml. thioglycollic acid, 5.0 mg. resazurin and 10g. agar were added per litre of tap water and the pH adjusted to 7.0.

For the description of colonial morphology surface cultures were prepared in 6, using Petri dishes and the McIntosh and Fildes jar. Oxygen requirements were determined by shake cultures in 6. All the strains discussed in the following sections have been found to be obligate anaerobes. The production of indole and related substances was investigated in 10. Tests were conducted on cultures of different ages, using Ehrlich's reagents and also vanillin and hydrochloric acid with the technique described by Roessler & McClung (1943). Ability to ferment test substances was determined by a comparison of growth, gas production and change in pH with that occurring in a control tube containing no added substance. Seals of 12 were poured on top of the inoculated medium and their rise served as a measurement of gas production. Growth was judged by inspection and change in pH by withdrawing part of the culture with a Pasteur pipette and testing with brom-cresol-purple (brom-thymol-blue in the case of lactate) on a spot-plate/

spot-plate. Ability to ferment starch was tested for by withdrawing part of the culture to a spot-plate and adding a few drops of Gram's iodine.

Volatile acids liberated during the course of metabolism were identified as follows. 25 ml. of the fermentation medium containing 1% carbohydrate were inoculated and after five days incubation the culture was shaken with a few ml. of ether. The ether was then mixed with 1 ml. of water and permitted to evaporate. The volatile acids remaining in solution in the water were separated and identified by paper chromatography (p.59).

Lactate attacking species

Type 1.

Morphology: The vegetative cells are rod-shaped, largish and occur singly and in pairs. The Gram stain is positive with young cultures and negative with older cultures. The cells readily tend to become distended into uneven shapes and to take up stains unevenly. The organism is motile. When grown in starch or glucose media, iodophilic compounds are stored. Oval spores are formed, almost always in a subterminal position, and produce a swelling of the rod.

Surface colonies: These are creamy white, opaque and circular. The surface is raised, smooth and shiny and the margin entire.

Coagulated blood: Reddening, but no digestion or blackening is produced.

Iron-glucose-gelatin: No liquefaction or blackening (after 12 days' incubation).

Iron/

Iron-litmus-milk: A gas-torn acid clot is formed within 1 - 2 days.

Corn-liver mash: The starch gel is rapidly digested with vigorous evolution of gas except with strains unable to ferment starch.

Indole: Not formed.

Fermentation of carbohydrates: Xylose, glucose, fructose, galactose, mannose, lactose, maltose and inulin are fermented. Starch is fermented with rare exceptions. Arabinose is attacked by the majority of strains tested. The fermentation of mannitol, sorbitol, and glycerin is variable. Rhamnose is attacked by a minority of strains tested. In all cases where carbohydrates were attacked, acid and gas were liberated. 1% sodium lactate, when added to medium 11 was not fermented. In the presence of acetate this compound was readily dissimilated with rare exceptions (see below).

Fermentation products: Butyric acid is produced from glucose and lactate plus acetate.

Temperature of incubation: The small number of strains tested initiated growth more quickly and liberated larger volumes of gas at 37° than at 22° or 30°. No growth was obtained at 45°.

During the whole course of the work, forty-five pure cultures of this type were isolated. All cultures were examined for size, shape and staining properties of vegetative cells, the type of sporulation, ability to liquefy gelatin, storage of iodophilic compounds, fermentation of lactate in the presence of acetate and fermentation of starch as judged by growth/

growth and gas production in corn-liver mash. Motility and fermentation of starch in medium 11 was determined for a smaller number of strains. All other tests were conducted with six representative cultures.

Thirteen of the forty-five strains isolated were obtained from primary cultures in the selective lactate medium and thirty-two by methods not selective for lactate attackers (selective gelatin medium and the technique described on p.34). Two of the latter thirty-two strains did not ferment lactate in the presence of acetate. It appears that, although common to the majority of strains of this type, the fermentation of lactate is not a universal characteristic.

Type 1 appears to be identical with the anaerobes described by Bhat & Barker (1947) and named by these authors Cl. lacto-acetophilum. As stated by Bhat & Barker, the relationship of Cl. lacto-acetophilum to Cl. butyricum and its sub-species as defined in Bergey's Manual (Breed, Murray & Hitchens, 1948) is very close. The operative difference is that Cl. butyricum and sub-species are not considered to be able to ferment lactate. On closer examination, this difference tends to disappear. Lactate attacking strains in pure culture cannot ferment lactate as such; special conditions are required for the fermentation to proceed. The evidence suggests that if the cultures on which the classification in Bergey's Manual is based had been tested in the presence of acetate, lactate would in all probability have been attacked. There appears to be a good case for broadening the definition of the species/

species Cl. butyricum to include strains capable of attacking lactate in the presence of acetate.

Richard (1948, 1950) has put forward the view that variability in morphological and cultural characters of butyric acid bacteria is such that no differentiation into species is possible. The scope of the work done for this thesis has not been sufficient to permit conclusions to be drawn on this aspect. No signs of variability have yet been found in what were considered to be diagnostic characters of type 1 viz. morphology in corn-liver mash, motility, storage of iodophilic compounds, type of sporulation, inability to liquefy gelatine, and fermentation of a wide range of carbohydrates.

Type 2

Morphology: The vegetative cells are rodshaped, of medium size, and occur singly and in pairs. Involution forms and filaments appear in old cultures. The Gram stain is positive in young cultures and becomes negative with increasing age. Motility has so far not been detected. Cells grown in corn-liver mash or medium 4 and examined microscopically in wet mounts plus iodine at times show faint blue colouration. The storage of iodophilic compounds, if such takes place, is not comparable in extent with that shown by strains of type 1. Oval spores are formed, almost always in a terminal position.

Surface colonies: These are white, opaque and circular. The surface is slightly raised with a raised centre, and appears moist and shiny. Fingerlike outgrowths often appear.

Coagulated blood: Reddening, but no digestion or blackening.

Iron-/

Iron-glucose-gelatin: No liquefaction or blackening is produced (after 12 days incubation).

Iron-litmus-milk: Acidified but not clotted (after 12 days incubation).

Corn-liver mash: The majority of strains initiate no change apart from the production of a small amount of gas. Starch-fermenting strains digest the gel but without extensive evolution of gas.

Indole: Not formed.

Fermentation of carbohydrates: Glucose, fructose, galactose, mannose, lactose and glycerin are fermented. Maltose is fermented by the majority of strains tested. The fermentation of arabinose and starch is variable. Xylose, rhamnose, mannitol, sorbitol, and inulin are not attacked. Lactate is dissimilated in the presence of acetate but not otherwise. Acid and gas were producing during the fermentation of carbohydrates.

Fermentation products: Butyric acid is produced from glucose and lactate plus acetate.

Temperature: Among the few strains tested, growth was initiated more quickly and larger amounts of gas liberated at 37° than at 22° or 30°. The organisms were further able to grow at 45° but not at 50°.

Thirty-six pure cultures conforming to this type were isolated from ensiled grass. All cultures were examined for size, shape and staining properties of vegetative cells, the type of sporulation, ability to liquefy gelatin, storage of iodophilic compounds, fermentation of lactate in the presence of/

of acetate and fermentation of starch as judged by growth and gas production in corn-liver mash. Motility was determined for a smaller number of strains and the remaining tests were conducted with four representative cultures.

Type 2, as described above, does not correspond well with any of the species of the genus Clostridium described in Bergey's Manual (Breed, Murray & Hitchens, 1948). Organisms closely related to Cl. butyricum but which form terminal spores have been described (McCoy, Fred, Peterson & Hastings, 1930). Such strains, however, liquefy gelatin and give a strong granulose reaction. The species to which type 2 appears to be most closely related is Cl. paraputrificum (Bienstock) Snyder. The main difference is that motility has not yet been demonstrated in type 2. In this connection it may be pointed out that Hall & Snyder (1934) have found only a small proportion of the rods to be motile in cultures of Cl. paraputrificum. No records of the action of Cl. paraputrificum on lactate in the presence of acetate are available.

Type 3.

Morphology: The vegetative cells are rodshaped, large and occur singly and in pairs. The cells do not tend to become distended into irregular shapes. The Gram stain is positive and this staining property is not lost quickly. The organisms are motile. Iodophilic compounds are stored when cultures are prepared in medium 4, but not in corn-liver mash. Oval spores are formed, generally in a sub-terminal position. The spores produce/

produce a slight swelling of the rod.

Surface colonies: These are white, opaque, and circular. The surface is raised, smooth and shiny. The margin is entire.

Coagulated blood: No change.

Iron-glucose-gelatin: No liquefaction or blackening is produced (after 12 days incubation).

Iron-litmus-milk: No change (after 12 days incubation).

Corn-liver mash: A small amount of gas is formed.

Indole: Not formed.

Fermentation of carbohydrates: Glucose and fructose are fermented. Of the strains tested one attacked xylose and another arabinose. Galactose, mannose, maltose, lactose, starch, inulin, sorbitol, mannitol, rhamnose and glycerine are not fermented. Lactate is attacked in the presence of acetate, but not otherwise. Acid and gas is produced during the fermentation of carbohydrates.

Temperature: Among the strains tested, growth was initiated more quickly at 37° than at 30° or 22°. No growth was obtained at 45°.

Fourteen strains belonging to type 3 were isolated from ensiled grass. The morphology of vegetative cells (including motility and granulose reaction), sporulation, liquefaction of gelatin, fermentation of lactate in the presence of acetate, and fermentation of starch in corn-liver mash were examined for all strains. Other tests were conducted with six representative cultures.

Type 3 differs from type 1 in its limited capabilities of utilising/

utilising carbohydrates. Further differences are found in morphology and in that growth of type 3 is decidedly slower in appearing on all the media tried. In the small number of carbohydrates fermented, type 3 resembles the lactate-attacking anaerobe, Cl. tyrobutyricum described by van Beynum & Pette (1935 - 36). Two strains of Cl. tyrobutyricum were kindly supplied by Dr. Pette. Examination of their morphological and cultural characters showed that these were identical with the characters of type 3.

The formation of the species Cl. tyrobutyricum to accommodate lactate fermenting butyric acid bacteria has been criticised by Burri (1936) and Richard (1948, 1950). In the sixth edition of Bergey's Manual, Cl. tyrobutyricum is given as a synonym for Cl. butyricum Prazmowski. In so far as it appears that almost all strains of butyric acid bacteria have the ability to attack lactate, the latter authors seem to be justified in their criticism. Yet, Cl. tyrobutyricum (= type 3) differs in other respects, such as morphology and fermentation pattern, and in this work the differences have, up to the present, proved to be constant. The evidence suggests that the separation of Cl. tyrobutyricum from Cl. butyricum and its establishment as a species is feasible, should this prove to be in the best interests of classification.

Type 4.

Morphology: The vegetative cells are small, slender rods; the Gram stain is predominantly negative. The organism is motile. Circular spores are formed in a terminal position.

Coagulated/

Coagulated blood: No change.

Iron-glucose-gelatin: No blackening or liquefaction is produced (after 12 days incubation).

Iron-litmus-milk: A small amount of gas is formed (during 12 days incubation).

Indole: Not formed.

Fermentation of carbohydrates: Glucose is fermented. The fermentation of mannitol is variable. Lactose and starch are not fermented. Lactate is fermented in the presence of acetate but not otherwise.

Fermentation products: Butyric and caproic acids are formed during the dissimilation of lactate plus acetate.

Two strains corresponding to type 4 have been isolated. The relationship of these organisms to species described in the literature is not yet clear.

Type 5.

Morphology: The cells are spherical, small, and occur singly, in pairs and large irregular clusters. The Gram stain is negative.

Surface colonies: These are whitish, translucent and circular. The surface is raised and smooth and the margin entire.

Corn-liver mash: Gas is formed.

Fermentation of carbohydrates: Glucose and fructose are not fermented. Lactate is attacked in the presence or absence of acetate.

Fermentation products: Propionic acid is formed during the fermentation/

fermentation of lactate.

Two strains of this type have been isolated. From the above characters and within the scope of the work done they appear identical with the anaerobes described by Johns (1951) and identified by him as Veillonella gazogenes (Hall & Howitt) Murray.

Proteolytic species

Cl. bifermentans

Morphology: The vegetative cells are rodshaped, large and occur singly, in pairs and short chains. The Gram stain is positive, becoming negative with increasing age. The organism is motile. Oval spores are formed, and are placed centrally or subterminally. The spores do not distinctly swell the rods.

Surface colonies: These are whitish, translucent and circular. The surface is slightly raised, smooth and shiny. The margin is entire.

Coagulated blood: Blackening and digestion are produced.

Iron-glucose-gelatin: Liquefaction and blackening are produced.

Iron-litmus-milk: The milk is cleared and blackened.

Indole: Indole is formed.

Fermentation of carbohydrates: Glucose, fructose and maltose are fermented. Lactose, inulin and lactate with or without acetate are not fermented. Acid and gas were produced during the dissimilation of carbohydrates.

Eighteen strains of this type have been isolated from ensiled grass. All strains were examined for morphology of vegetative/

vegetative cells, type of sporulation, liquefaction of gelatin, production of indole and fermentation of lactate in the presence of acetate. Other tests were conducted on three representative cultures. The characters described above indicate that the strains belong to the species Cl. bifermentans (Weinberg & Seguin) Bergey et alia.

Cl. sporogenes

Morphology: The vegetative cells are rodshaped, of medium size and occur singly and in pairs. The Gram stain is positive, becoming negative with increasing age. The organisms are motile. Oval subterminal spores are formed and produce swelling of the rod. In some strains the spores are predominantly terminal.

Surface colonies: These are white, opaque, and have a pronounced tendency to spread. Root-like outgrowths frequently appear.

Coagulated blood: Blackening and digestion are produced.

Iron-glucose-gelatin: Liquefaction and blackening are produced.

Iron-litmus-milk: The milk is cleared and blackened.

Indole: Not formed. A substance is produced which gives a violet colour with vanillin and hydrochloric acid.

Fermentation of carbohydrates: Glucose, fructose and maltose are fermented. Mannitol, inulin, lactose and lactate with or without acetate are not fermented. Acid and gas were produced during the dissimilation of carbohydrates.

Thirty-two strains of this type have been isolated from ensiled/

ensiled grass. All cultures were examined for morphology of vegetative cells, type of sporulation, liquefaction of gelatin, production of indole or related compounds, and fermentation of lactate in the presence of acetate. Other tests were conducted on eight representative cultures. The characters of the strains examined show that they belong to the species Cl. sporogenes (Metchnikoff) Bergey et alia.

Cl. welchii

Morphology: The vegetative cells are rodshaped, large and occur singly and in pairs. The Gram stain is positive and this staining property is not lost quickly. Motility has not been observed. Spores have not been observed. Iodophilic compounds are not stored by cells grown in corn-liver mash.

Surface colonies: These are white, circular or nearly so and opaque. The surface is raised, smooth and shiny. The margin is entire.

Coagulated blood: Reddening, but no digestion or blackening are produced.

Iron-glucose-gelatin: Liquefaction and a slight darkening are produced.

Iron-litmus-milk: A gas-torn acid clot is formed within one day.

Indole: Not formed.

Fermentation of carbohydrates: Glucose, fructose, galactose, mannose, maltose, lactose, inulin, starch and glycerin are fermented. Arabinose, xylose, sorbitol, mannitol, rhamnose and lactate with or without acetate are not fermented. Acid and/

and gas were produced during the dissimilation of carbohydrates.

Seven strains of this type have been isolated from ensiled grass. All cultures were examined for morphology of vegetative cells, type of sporulation, storage of iodophilic compounds, motility, liquefaction of gelatin, fermentation of starch in corn-liver mash, and fermentation of lactate in the presence of acetate. Other tests were conducted with one strain. The characters described above indicate the strains belong to the species Cl. welchii (Wilson & Miles, 1946).

Anaerobes not liquefying gelatin or able to ferment lactate

Type 6.

Morphology: The vegetative cells are rods, small and fusiform in shape. They occur singly and often in pairs. The Gram stain is predominantly negative. Some strains store iodophilic compounds, others do not. Granulose-bearing cells of type 6 can be differentiated from type 1 by differences in shape and size. The organisms are motile. Spherical spores are formed and appear in a subterminal or terminal position.

Coagulated blood: Reddening, but no blackening or digestion.

Iron-glucose-gelatin: No blackening or liquefaction is produced (after 12 days incubation).

Iron-litmus-milk: The milk is acidified, at times clotted (after 12 days incubation).

Indole: Produced by some strains, but not by others.

Fermentation of carbohydrates: Arabinose, xylose, glucose, fructose, galactose, mannose, rhamnose, lactose, maltose and starch fermented. The fermentation of mannitol is variable.

Inulin,/

Inulin, sorbitol, glycerin and lactate with or without acetate are not fermented. Acid and gas were produced during the dissimilation of carbohydrates.

Eight strains of this type have been isolated from ensiled grass. All cultures were examined for the morphology of vegetative cells, ability to store iodophilic compounds, type of sporulation, ability to liquefy gelatin, production of indole and fermentation of lactate in the presence of acetate. Other tests were conducted with three cultures. The characters described above agree closely with those given in Bergey's Manual (Breed, Murray & Hitchens, 1948) for Cl. sphenoides (Bulloch et alia) Bergey et alia.

Type 7.

Morphology: The vegetative cells are rods, of medium size and staining Gram negative.

Coagulated blood: Reddening, but no blackening or digestion.

Iron-glucose-gelatin: No blackening or liquefaction is produced (after 12 days incubation).

Iron-litmus-milk: No change (after 12 days incubation).

Indole: This is not formed. A substance giving a violet colour with vanillin and hydrochloric acid is produced.

Fermentation of carbohydrates: Glucose is fermented. Lactose, starch, mannitol and lactate with or without acetate are not attacked.

Distinctive character: A peculiar foul smell is produced in all media tested.

One strain of this type has been isolated. Its relationship to/

to species described in the literature is not yet clear.

Type 8.

Morphology: The vegetative cells are rods of medium size. The Gram stain is positive in young cultures and negative in older cultures. Spherical spores are formed in a terminal or almost terminal position. The spores produce a swelling of the rod.

Coagulated blood: Reddening but no blackening or digestion.

Iron-glucose-gelatin : No blackening or liquefaction is produced (after 12 days incubation).

Iron-litmus-milk: Reduction of the litmus but no other change (after 12 days incubation).

Indole: Indole is formed.

Fermentation of carbohydrates: The fermentation of glucose and fructose is variable. Lactose, starch, mannitol and lactate with or without acetate are not fermented. Acid and gas were liberated during the fermentations.

Five strains of this type have been isolated. All cultures were examined for morphology of vegetative cells, type of sporulation, gelatin liquefaction, production of indole and fermentation of lactate in the presence of acetate. Other tests were conducted with three cultures. The relationship of type 8 with strains described in the literature is not yet clear.

Type 9.

Morphology: The vegetative cells are long, slender rods, staining/

TABLE 22.

Dry matter and crude protein content of fresh grass and plan of experiments 7 - 10.

	Experiments			
	7	8	9	10
Dry matter %				
Fresh grass	21.1	21.4	25.6	21.0
Grass + water	13.6	19.6	15.4	20.2
Wilted grass	34.4	28.1	41.2	30.0
Wilted + water	21.2	16.8	24.7	18.0
Crude protein (% of dry matter)	14	8.11	9.03	10.75
Treatments	22°	22°	22°	22°
	40°	40°	40°	40°
	Uncut	Uncut	Uncut	Uncut
	Minced	Minced	Minced	Minced
	+ Water	+ Water	+ Water	+ Water
	Wilted	Wilted	Wilted	Wilted
	Wilted + water	Wilted + water + lactobacilli	Wilted + water + lactobacilli	Wilted + water + lactobacilli
	30°	30°	30°	30°

No. of tubes prepared for each treatment : 5
Age of silages on examination : 1, 2, 3, 8 and 180 days.

staining Gram positive when young and negative in older cultures. The spores are oval, terminal and swell the rod.

Coagulated blood: Reddened, but not digested or blackened.

Iron-glucose-gelatin: No blackening or liquefaction (after 12 days incubation).

Iron-litmus-milk: An acid clot is formed in three to five days and a small amount of gas produced.

Indole: Not formed.

Fermentation of carbohydrates: Glucose, lactose, mannitol and starch are fermented. Lactate with or without acetate is not attacked. Acid and gas is formed during the dissimilation of carbohydrates.

Two such strains have been isolated. Tests were conducted with both cultures. The relationship to anaerobes described in the literature is not yet clear.

Bacteriological results of experiments with test-tube silages (expts. 7 - 10)

The dry matter and crude protein content of the grass before ensilage and a plan of the experiments are given in Table 22. The complete results of the bacteriological work are given in the appendix (Tables 45-8) while summaries and relevant extracts are placed in the text. The results obtained for experiment 8 were incomplete due to temporary technical difficulties.

Anaerobic/

TABLE 23.

Anaerobic flora of grass prior to ensilage

Expt.	Lactate medium					Gelatin medium				
	Dilution count[1] end-points				Species	Dilution count[1] end-points				Species
	1/12	1/120	1/1,200	1/12,000		1/12	1/120	1/1,200	1/12,000	
7		---	---	---			---	---	---	
8	---	---	---			---	---	---		
9	---	---	---			+++	+++	+--	---	Cl. bifermentans & Cl. welchii
10	++-	---	---		Type 1	++-	---	---		Cl. bifermentans

[1] 3 replicates inoculated from each dilution.

TABLE 24.

Species of anaerobes found to have multiplied in experiments 7 - 10 and frequency of occurrence of the different types

No. of silages out of a total of 157 in which the following were detected by isolations and other means (see p.58) in dilutions of 1:12,000 or higher.

Type 1	Type 2	Type 3	Type 5	Type 6	Type 8	Cl. sporogenes	Cl. bifermentans	Cl. welchii
30	20	4	2	5	2	17	40	4

Anaerobic flora prior to ensilage

As shown in Table 23, numbers of anaerobes on the fresh material were low. Differences in numbers and in the species present appeared, but their significance, if any, is obscure. Low numbers of anaerobes on fresh grass and green crops have been found by Scheunert & Schieblich (1926) and Allen & Harrison (1937a).

Species of anaerobes which initiated multiplication

Anaerobes were considered to have multiplied in the silages if their numbers exceeded an arbitrary limit viz. if they were present in dilutions of 1 : 12,000 or higher. This approach, instead of direct comparisons with counts on the fresh grass, was employed because of the dangers of sampling errors and the relative inaccuracy of dilution counts. In Table 24, the species which were detected in such dilutions and the frequency with which they were found is given. It must be noted that identification of species was not carried out in all cases where growth of anaerobes occurred in the selective media (see Appendix, Tables 45-8).

Comparing these results with findings in the literature (Introduction p. 7), it may be seen that there is agreement concerning the importance of type 1 (= Cl. butyricum) and Cl. sporogenes and to a lesser extent of Cl. bifermentans. Cl. bifermentans was found less frequently than Cl. butyricum or Cl. sporogenes by Scheunert & Schieblich (1926) and not at all by Allen & Harrison (1937a).

A notable difference with respect to previous work is the frequent/

frequent occurrence of type 2 in the test-tube silages. No species corresponding to this organism has been described in the literature examined as occurring in ensiled fodder. Type 3, identical with Cl. tyrobutyricum was isolated on rare occasions in this part of the work. Van Beynum & Pette (1935 - 36, 1936) found Cl. tyrobutyricum to be widespread in ensiled materials. It may be noted in this connection that in two farm silages (p. 96) type 3 was among the dominant anaerobic bacteria. This species may have a much wider distribution than indicated by the results in Table 24.

Of the other types, all of which were found infrequently, only Cl. welchii has already been described as occurring in ensiled materials (Allen & Harrison, 1937a). Type 5 is of interest as propionic acid is liberated during the course of its metabolism. Propionic acid has been found in ensiled fodder (Virtanen & Miettinen, 1951). Any conclusions drawn about the importance of these infrequently occurring species must be qualified by the fact that media selective for lactate-fermenting and proteolytic anaerobes were employed. The role played by strains of type 1 unable to ferment lactate (p. 65), two such strains having been isolated from the test-tube silages, should also be considered from such a point of view. However, anaerobes unable to attack lactate or gelatin found conditions in the gelatin medium suitable for growth and frequent isolations on non-selective media were made from the gelatin medium. The evidence as a whole, therefore, suggests that they play a minor role.

Time/

FIG. 9 TIME AND EXTENT OF MULTIPLICATION OF ANAEROBES.

X = HIGHEST DILUTION IN WHICH ANAEROBES WERE DETECTED IN A PARTICULAR SILAGE.

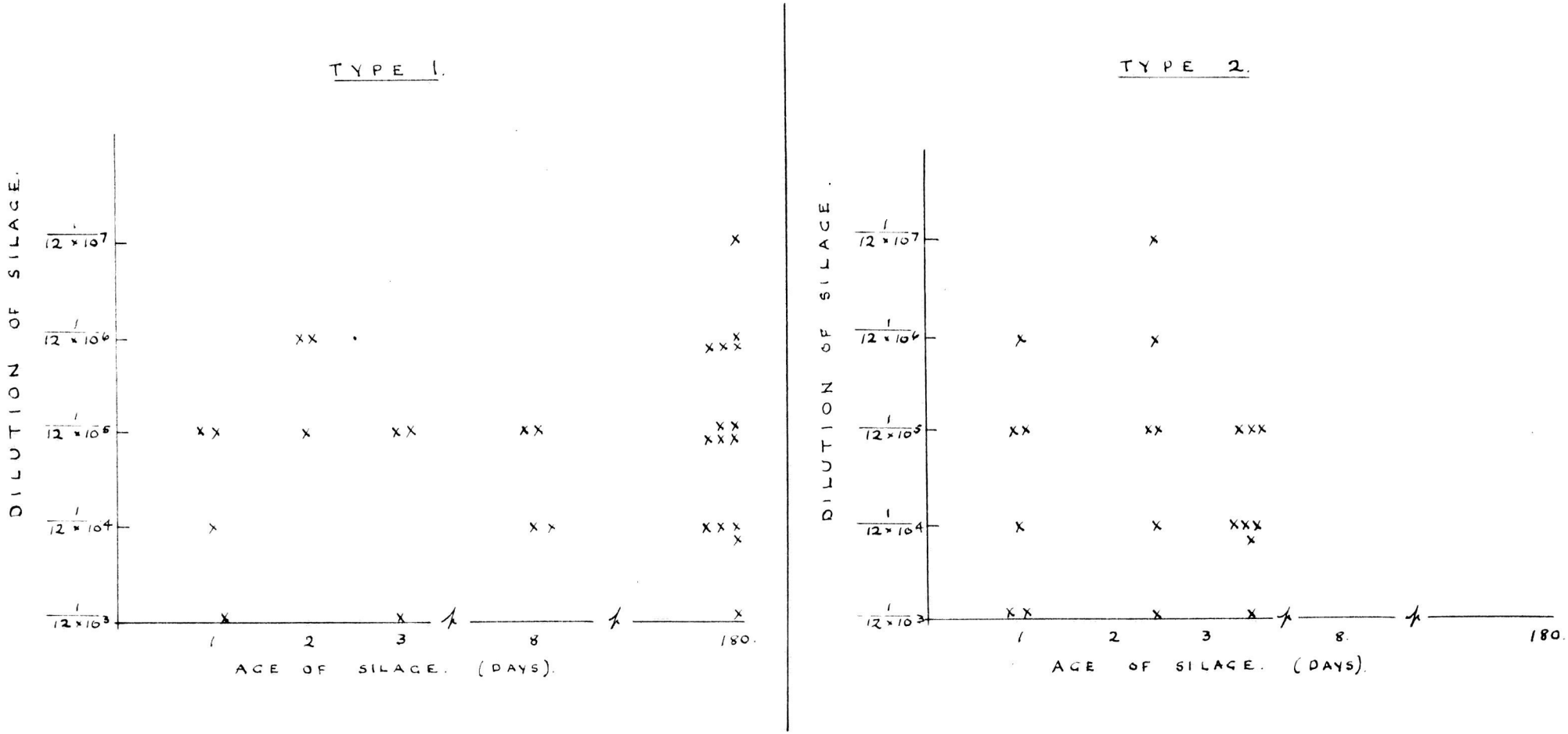


FIG.10. TIME AND EXTENT OF MULTIPLICATION OF ANAEROBES.

X = HIGHEST DILUTION IN WHICH ANAEROBES WERE DETECTED IN A PARTICULAR SILAGE.

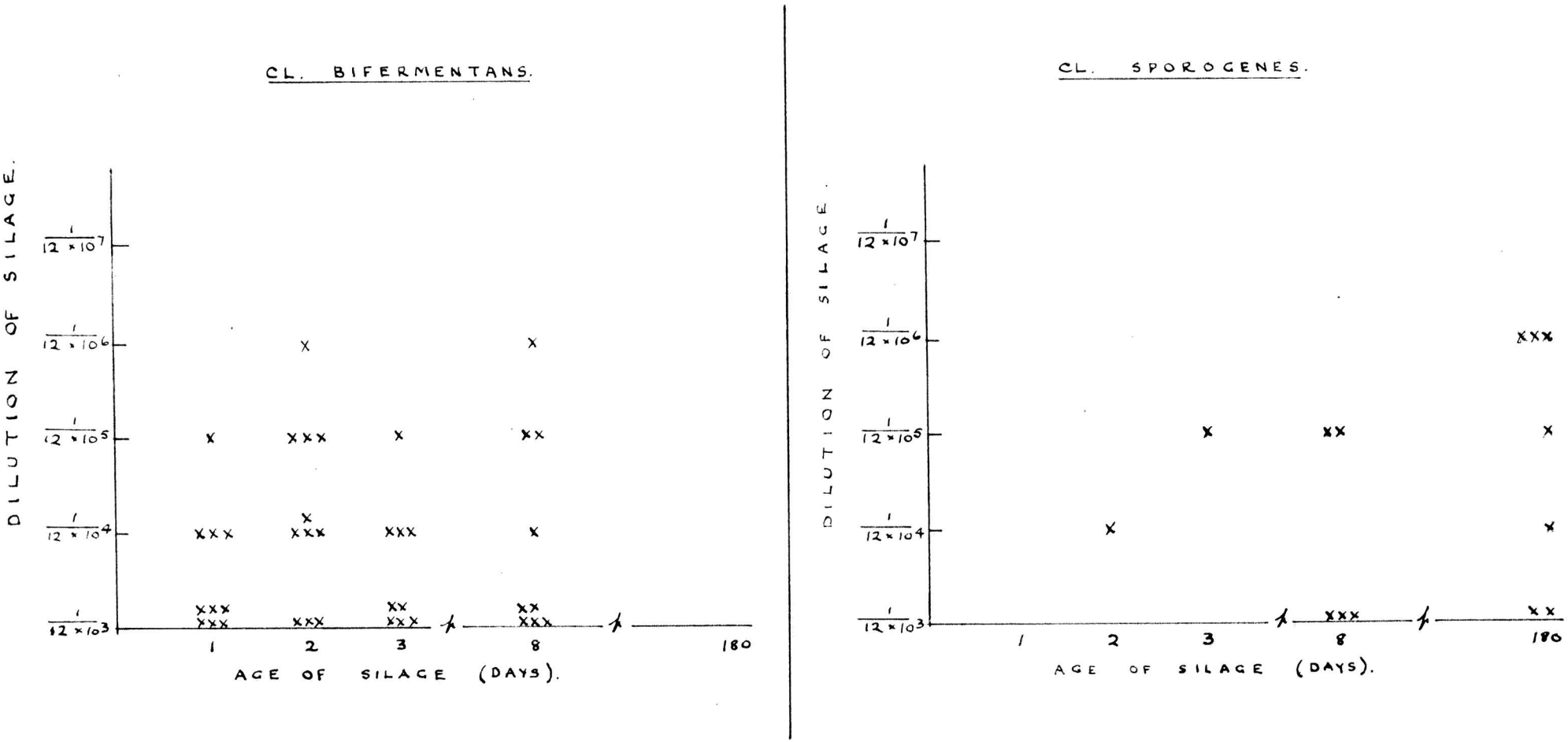


TABLE 25.

Time and extent of multiplication of anaerobes

Species	Age of silage (days)				
	1	2	3	8	180
Cl. welchii	$\frac{1}{12 \times 10^4}$ (1) $\frac{1}{12 \times 10^5}$ (2)	$\frac{1}{12 \times 10^5}$ (1)	-	-	-
Type 3	-	-	-	$\frac{1}{12 \times 10^5}$ (1) $\frac{1}{12 \times 10^5}$ (1)	$\frac{1}{12 \times 10^5}$ (2)
Type 5	-	$\frac{}{12 \times 10^4}$ (1)	$\frac{}{12 \times 10^4}$ (1)	-	-
Type 6	$\frac{1}{12 \times 10^4}$ (1)	$\frac{1}{12 \times 10^5}$ (1)	$\frac{1}{12 \times 10^4}$ (1)	$\frac{1}{12 \times 10^4}$ (1)	$\frac{1}{12 \times 10^5}$ (1)
Type 8	$\frac{1}{12 \times 10^4}$ (1)	-	-	-	$\frac{1}{12 \times 10^4}$ (1)

Dilutions represent the highest dilution in which anaerobes were detected in a particular silage.

Figures in brackets represent numbers of silages in which the species was found.

conclusions can be drawn about the multiplication of the other types isolated. What evidence has been obtained indicates that Cl. welchii proliferated in the early stages only and that the reverse is true of type 3.

Results published by other workers with which the above findings can be compared are few. Scheunert & Schieblich (1926) carried out the majority of their detailed investigations on silages in which the temperature had risen to 50°C or higher in the early stages. As might be expected, the mesophilic species with which they were dealing did not multiply until cooling set in. Allen & Harrison (1937a, b) stated that in their silages numbers of anaerobes remained low and reached a peak 10-15 days for the first few days after ensiling. This information, however, appears to apply solely to Cl. sporogenes. Olsen (1951), using unheated material and a modern technique, found an increase in numbers of Cl. butyricum in ensiled sugar-beet pulp after three days and further increases up to twelve days. Thereafter numbers remained more or less constant up to sixty-nine days.

Van Beynum & Pette (1936) came to the conclusion that growth of Cl. butyricum will tend to take place in the early stages of the ensilage process, when there is still a plentiful supply of carbohydrates available. This conclusion was based on the mistaken assumption that Cl. butyricum cannot attack lactate. They considered on the other hand that multiplication of the lactate fermenting Cl. tyrobutyricum would tend to be dependant on the previous production of lactic acid, as this species has very limited abilities to attack carbohydrates.

Of/

TABLE 26.

Comparison of dilution counts on heated and unheated silage

Silages of experiment 8 after 6 month's incubation

Treatment	Most probable number per g. dry matter			
	Pasteurised[1]		Unpasteurised	
	Lactate medium	Gelatin medium	Lactate medium	Gelatin medium
22°	1.8×10^5	$<6 \times 10^4$	2.6×10^6	$<6 \times 10^4$
40°	$<6 \times 10^4$	$<6 \times 10^4$	$<6 \times 10^4$	$<6 \times 10^4$
Uncut	4.5×10^6	$<6 \times 10^4$	2.6×10^7	$<6 \times 10^4$
Minced	$<6 \times 10^4$	$<6 \times 10^4$	$<6 \times 10^4$	$<6 \times 10^4$
+ water	1.2×10^5	$<6 \times 10^4$	2.6×10^6	$<6 \times 10^4$
Wilted	6×10^4	2.5×10^6	5.6×10^5	1.4×10^6
Wilted + water	1.2×10^7	$<6 \times 10^4$	4.5×10^7	$<6 \times 10^4$
+ lactobacilli	$<6 \times 10^4$	$<6 \times 10^4$	$<6 \times 10^4$	$<6 \times 10^4$
30° [2]	5.5×10^8	1.2×10^7	2.6×10^8	1.4×10^8

Error of most probable number at confidence interval of
1 : $33\frac{1}{3}$ = 487% to 20.5%

[1] 70° for 15 min.
[2] Mould development occurred in this tube.

TABLE 27.

Effect of pH on the multiplication of anaerobes

Lactate fermenters

pH of silage

4.0	4.0 - 4.2	4.2 - 4.4	4.4 - 4.6	4.6 - 4.8	4.8 - 5.0	5.0
1	4	2	6	8	9	43

Proteolytic anaerobes

pH of silage

4.6	4.6 - 4.8	4.8 - 5.0	5.0 - 5.2	5.2 - 5.4	5.4
2	4	7	7	6	31

Figures represent the no. of silages having a particular pH in which anaerobes were detected by the use of selective media and certain tests (p. 58) in dilutions of 1 : 12,000 or higher.

Of interest in this connection is the data (Table 25) suggesting that growth of type 3 commenced in the later stages of the fermentation. The evidence collected suggests that anaerobes may be more active in the early stages of the fermentation than was previously thought to be the case. Further, it appears that examination of mature silages supplies no reliable information on the activity of certain species.

Comparisons between counts on heated and unheated material were made on a small number of silages. The results are presented in Table 26. Most probable numbers as calculated from the unheated silage were significantly greater than those from pasteurised silage in five cases. In three other instances there was no significant difference. In no case, however, were the differences great. It appears likely that after six months' incubation the majority of viable cells were present in the form of spores.

The stage of the fermentation at which inactive spores are formed and the percentage of cells sporulating must necessarily be reflected in the changes produced by anaerobes in ensiled fodder. Further investigations of this problem seems therefore to be desirable.

The effect of pH on the multiplication of anaerobes

Low pH levels exerted an inhibitory influence on the multiplication of both lactate fermenting and proteolytic anaerobes (Table 27). In silages having pH levels of less than 4.4 lactate attackers were only rarely found, as were proteolytic species when the pH had dropped to below 4.8. The data/

data suggests that anaerobes were not multiplying at the low pH values, but had reached high numbers at a previous stage under more favourable conditions, e.g. expt. 9 + water and expt. 10 + lactobacilli (Tables 47-8 p. 108-9). Spores which had been formed at an earlier period may be expected to survive in acid conditions.

These results are in accordance with the main body of evidence in the literature concerning the pH tolerance of anaerobes (Introduction p. 12). From time to time conflicting reports have been made, such as Olsen (1951) who found multiplication of butyric acid bacteria in ensiled sugar-beet pulp of pH 4.0, and Allen & Harrison (1937b) in whose experiments Cl. sporogenes proliferated in silages with a pH varying between 3.9 and 4.7. No indication has been obtained of the presence of such aciduric strains in the test-tube silages.

The effect of variation in temperature

In this and other sections dealing with the effect of variation in certain factors, the 30° treatment (Table 21, p. 57) will be taken as the standard against which other treatments are compared. It occupies a central position between the extremes.

To assist in the determination of significant differences between treatments and the standard, use will be made of most probable numbers and their estimated error (48% to 20.5%). It is clear, however, that in this case more than the accuracy of the method of enumeration has to be considered.

The/

TABLE 28.

Effect of temperature on the multiplication of lactate fermenters

Expt.	Treatment	Age of silage (days)				
		1	2	3	8	180
7	Standard[1]	<0.6	18	48	120	9
	40° [2]	>930	770	12	<0.5	<6.6
	22° [2]	100[3]	3.3	0.1	12	2770
8	Standard[1]	0.1				26,00*
	40° [2]	26,000				<0.02
	22° [2]	<6				1
9	Standard[1]	<0.6	1.4	2.3	1.4	0.06
	40° [2]	100[3]	<30	<20	<30	100[3]
	22° [2]	100[3]	<30	4	7	$2^3 \times 10^4$
10	Standard[1]	26	5.6	0.06	0.06	<0.6
	40° [2]	10	46	4300	100[3]	100[3]
	22° [2]	<2	<10	100[3]	100[3]	100[3] *

1 Most probable numbers ~~per~~ 10^5

2 Count (most probable numbers) expressed as % of count of standard

3 Counts in treatment and standard both $<0.6 \times 10^5$

* Mould development occurred in this tube

TABLE 29.

Effect of temperature on the multiplication of proteolytic anaerobes

Expt.	Treatment	Age of silage (days)				
		1	2	3	8	180
7	Standard[1]	<0.6	24	120	90	240
	40° 2	23,000	230	0.5	0.6	<0.25
	22° 2	100[3]	5.5	4.7	28.8	37.5
8	Standard[1]	26	14		0.6	260*
	40° 2	540	4.4		430	<0.23
	22° 2	<0.2	<4.3		4300	<0.23
9	Standard[1]	<0.6	<0.6	<0.6	<0.6	<0.6
	40° 2	100[3]	100[3]	>230	100[3]	100[3]
	22° 2	100[3]	100[3]	100[3]	100[3]	100[3]
10	Standard[1]	<0.6	<0.6	0.6	<0.6	<0.6
	40° 2	>100	100[3]	<100	100[3]	100[3]
	22° 2	100[3]	100[3]	<100	100[3]	100[3]*

1 Most probable numbers ~~per~~ $\div 10^5$

2 Count (most probable numbers) expressed as % of count of standard

3 Count in treatment and standard both $< 0.6 \times 10^5$

* Mould development occurred in this tube.

TABLE 30.

pH values of the silages in experiments 7 - 10

Expt.	Treatment	Time in days				
		1	2	3	8	180
7	22°C	6.54	6.46	6.18	6.10	5.24
	40°C	6.01	5.70	5.03	4.74	4.57
	Uncut	6.52	6.46	5.11	5.40	5.02
	Minced	5.58	4.48	4.30	4.36	4.52
	+ water	5.42	4.66	4.54	4.74	4.74
	Wilted	6.34	6.38	6.22	6.30	5.45
	Wilted + water	6.14	4.84	4.75	4.80	4.72
	Standard	6.38	6.28	5.89	5.74	5.26
8	22°C	6.66	6.22	6.22	5.66	4.97
	40°C	5.44	5.04	4.94	4.80	4.29
	Uncut	6.50	5.72	5.22	4.85	4.92
	Minced	6.02	4.77	4.60	4.32	4.29
	+ water	5.00	5.28	5.32	4.64	5.21
	Wilted	6.40	6.40	6.34	6.22	5.23
	Wilted + water	6.05	5.50	5.00	4.77	4.67
	+ lactobacilli	4.14	3.84	3.92	3.74	3.68
	Standard	6.50	5.33	5.23	5.08	7.03*
9	22°	6.24	6.00	6.27	6.14	5.14
	40°	6.10	5.78	5.48	5.38	4.30
	Uncut	6.11	6.22	6.11	5.72	4.58
	Minced	5.20	4.36	4.10	3.80	3.83
	+ water	5.86	4.95	4.10	4.32	3.98
	Wilted	6.02	6.07	5.91	5.82	5.27
	Wilted + water	6.15	5.72	4.92	5.09	4.20
	+ lactobacilli	4.32	4.14	3.96	3.78	3.74
	Standard	6.12	6.16	6.14	5.76	4.53
10	22°	6.30	5.77	5.80	4.88	5.32*
	40°	5.88	5.49	4.95	4.46	4.17
	Uncut	5.96	5.48	5.21	4.85	4.17
	Minced	4.65	4.28	4.18	4.20	3.95
	+ water	5.42	4.79	4.74	4.39	4.00
	Wilted	6.33	6.32	6.11	5.47	4.35
	Wilted + water	5.28	4.53	4.81	4.41	3.98
	+ lactobacilli	4.86	4.15	4.00	3.83	3.76
	Standard	5.90	5.14	5.06	4.59	4.16

* Mould development occurred in these tubes.

The silages have been prepared from grass taken from the same bulk sample but these smaller samples may still differ in certain respects. The amount of pressure exerted and air included when the grass was packed into tubes are further likely to vary somewhat, in spite of precautions taken. A wider margin of error than that calculated for the estimation of numbers in an even suspension by a dilution technique must therefore be allowed.

In Tables 28 and 29 numbers of anaerobes in the standard treatment are compared with numbers in silages incubated at 40° or 22°. For the latter silages the results are given as percentages of corresponding counts in the standard.

At 40° in experiment 7 and 8 lactate attackers multiplied more quickly than at 30°, but then the numbers dropped far below those of the standard. In experiment 9 these organisms did not develop at the higher temperature and in experiment 10 after eight days lactate fermenters were not present in dilutions of 1 : 12,000 at 40° or at 30°. The differences indicate, particularly in experiment 7, that the proliferation of lactate attacking anaerobes is to some extent inhibited at the higher temperature. Examination of Table 30 shows that in experiment 7, at least, the inhibition was not caused by low pH levels. Its cause is at present not clear, though it may be pointed out that anaerobes closely related to type 1 and not able to grow at 40° have been described (Tabachnick & Vaughn, 1948). Of the strains tested in this work, those belonging to types1 and 3 initiated growth at 37° but not at 45°, and

those/

TABLE 31.

Effect of degree of chopping on the multiplication of lactate fermenters

Expt.	Treatment	Age of silage (days)				
		1	2	3	8	180
7	Standard[1]	<0.6	18	48	120	9
	Uncut[2]	100[3]	13.3	250	7.5	2660
	Minced[2]	>2300	140	540	<0.5	300
8	Standard[1]	0.1				2600*
	Uncut[2]	300				10
	Minced[2]	5600				<0.023
9	Standard[1]	<0.6	1.4	2.3	1.4	0.06
	Uncut[2]	100[3]	<43	60.8	185	23,000
	Minced[2]	100[3]	<43	<26	<43	<100
10	Standard[1]	26	5.6	0.06	0.06	<0.6
	Uncut[2]	<2.3	161	900	4000	>200
	Minced[2]	0.23	<1	100[3]	100[3]	100[3]

1 Most probable numbers ~~per~~ 10^5

2 Counts (most probable numbers) expressed as % of count of standard

3 Counts in treatment and standard both $<0.6 \times 10^5$

* Mould development occurred in this tube

TABLE 32.

Effect of degree of chopping on the multiplication of proteolytic anaerobes

Expt.	Treatment	Age of silage (days)				
		1	2	3	8	180
7	Standard[1]	<0.6	24	120	90	240
	Uncut[2]	>400	100	20	133	100
	Minced[2]	100[3]	<2.5	<0.5	<0.6	<0.25
8	Standard[1]	26	14		0.6	160*
	Uncut[2]	<2.3	100		23,000	<0.23
	Minced[2]	<0.23	1000		<100	<0.23
9	Standard[1]	<0.6	<0.6	<0.6	<0.6	<0.6
	Uncut[2]	100[3]	100[3]	>230	>100	100[3]
	Minced[2]	100[3]	100[3]	100[3]	100[3]	100[3]
10	Standard[1]	<0.6	<0.6	0.6	<0.6	<0.6
	Uncut[2]	100[3]	>100	100	100[3]	100[3]
	Minced[2]	100[3]	100[3]	<100	100[3]	100[3]

1 Most probable numbers ~~per~~ $\div 10^5$

2 Count (most probable numbers) expressed as % of count of standard

3 Counts in treatment and standard both $<0.6 \times 10^5$

* Mould development occurred in this tube

those belonging to type 2 at both temperatures.

Proteolytic anaerobes in experiment 7 were present in higher numbers after one day at 40° than at 30°, but thereafter numbers fell off steeply at the higher temperature. The lower pH reached at 40° may explain the decline.

In the silages incubated at 22° anaerobes appeared to initiate growth more slowly than at 30°. Numbers, however, approached and at times exceeded those of the standard during the course of the incubation.

The effect of the degree of chopping

The relevant data are presented in Tables 31 and 32. Between the silages prepared from uncut grass and the standard, differences in the content of anaerobes tend to appear in isolated instances only, apart from numbers of lactate fermenters after six months incubation. These were higher in the uncut material in experiments 7, 9 and 10. The standard silage in experiment 8 at six months does not afford a fair comparison due to its abnormally high pH (Table 30, p.85).

After mincing, lactate attackers appeared to be able to initiate growth more quickly provided that the pH level did not fall rapidly (See Table 30). Of interest is the marked drop and subsequent rise in the count of these organisms at eight days in experiment 7. At this stage the pH had fallen to 4.3 and a reduction in numbers might be expected. Lactate fermenter type 3, however, appeared to find conditions suitable for growth and multiplied at some period between eight days and six months.

The/

TABLE 33.

Effect of moisture content on the multiplication of lactate fermenters

Expt.	Treatment	Age of Silage (days)					Moisture content (%)
		1	2	3	8	180	
7	Standard[1]	<0.6	18	48	120	9	78.9
	+ water[3]	>2660	170	330	60	170	86.4
	Wilted[3]	100[2]	<3.3	<1.2	0.5	<6.6	65.6
	Wilted + water[3]	> 200	<3.3	180	2.0	73	78.8
8	Standard[1]	0.1				2600*	78.6
	+ water[3]	6000				1	80.4
	Wilted[3]	<60				0.21	71.9
	Wilted + water[3]	60				17	83.2
9	Standard[1]	<0.6	1.4	2.3	1.4	0.06	74.4
	+ water[3]	15,000	6400	1000	3800	2000	84.6
	Wilted[3]	100[2]	<43	<26	<43	100[2]	58.8
	Wilted + water[3]	100[2]	64,000	500	1700	6000	75.3
10	Standard[1]	26	5.6	0.06	0.06	<0.6	79.0
	+ water[3]	530	160	15,000	100[2]	100[2]	79.8
	Wilted[3]	2.3	250	23,000	15,000	100[2]	70.0
	Wilted + water[3]	920	960	40,000	1000	100[2]	82.0

1 Most probable numbers $\div 10^5$
2 Counts in treatment and standard both $< 0.6 \times 10^5$
3 Count (most probable numbers) expressed as % of count of standard
* Mould development occurred in this tube

TABLE 34.

Effect of moisture content on the multiplication of proteolytic anaerobes

Expt.	Treatment	Age of silage (days)					Moisture Content (%)
		1	2	3	8	180	
7	Standard[1]	<0.6	24	120	90	240	78.9
	+ water[3]	100[2]	6.6	<0.5	8	0.03	86.4
	Wilted[3]	100[2]	<2.5	1.1	1.5	0.25	65.6
	Wilted + water[3]	>400	50	10	<0.6	<0.25	78.8
8	Standard[1]	26	14		0.6	260*	78.6
	+ water[3]	21	<4.3		230	<0.23	80.4
	Wilted[3]	0.7	10		23.000	5.3	71.9
	Wilted + water[3]	50	100		<100	<0.23	83.2
9	Standard[1]	<0.6	<0.6	<0.6	<0.6	<0.6	74.4
	+ water[3]	100[2]	100[2]	100[2]	>200	100[2]	84.6
	Wilted[3]	100[2]	100[2]	100[2]	100[2]	100[2]	58.8
	Wilted + water[3]	100[2]	100[2]	1002	100[2]	100[2]	75.3
10	Standard[1]	<0.6	<0.6	0.6	<0.6	<0.6	79.0
	+ water[3]	>430	>230	<100	100[2]	100[2]	79.8
	Wilted[3]	100[2]	100[2]	<100	100[2]	100[2]	70.0
	Wilted + water[3]	100[2]	100[2]	<100	>100	100[2]	82.0

1 Most probable numbers ~~per~~ 10^5

2 Counts in treatment and standard both $< 0.6 \times 10^5$

3 Count (most probable numbers) expressed as % of count of standard

* Mould development occurred in this tube.

The effect of changes in moisture content

When the moisture content of the grass was raised by the addition of water, lactate fermenters were able to initiate growth more quickly (Table 33). Even a small change in moisture content appeared to have this effect. In the later stages numbers corresponded quite closely to those in the standard in experiments 7 and 10 and were higher in experiment 9. Proliferation of proteolytic species was on the whole reduced by the addition of water (Table 34). The lower pH levels attained provide a likely explanation for this.

Wilting effectively reduced numbers of both types of anaerobes in experiment 7. In experiment 9 lactate fermenters were not detected in the wilted grass in dilutions of 1 : 12,000. In 10, on the other hand, counts of lactate fermenters were somewhat greater in the wilted grass than in the standard. The failure of anaerobes to initiate multiplication in the wilted material in experiment 7 was obviously not due to inhibition by a low pH (Table 30, p. 85). The fact that the numbers of lactate fermenters were restored to near those of the standard when water was added to the wilted grass suggests that the abstraction of water itself was in some way responsible. A similar effect may be noted from data on lactate fermenters in the wilted grass with and without addition of water in experiment 9. Proteolytic anaerobes did not appear to be stimulated to the same extent as saccharolytic species when water was added to wilted material. The tendency for lower pH values to be reached after the addition of water may, however,/

TABLE 35.

Effect of inoculation with lactobacilli on the multiplication of anaerobes

Lactate fermenters

Expt.	Treatment	Age of silage (days)				
		1	2	3	8	180
8	Standard[1]	0.1				2600
	+ lactobacilli[2]	<60				<0.02
9	Standard[1]	<0.6	1.4	2.3	1.4	0.06
	+ lactobacilli[2]	100[3]	<4.3	<26	<4.3	100[3]
10	Standard[1]	26	5.6	0.06	0.06	<0.6
	+ lactobacilli[2]	210	1000	100[3]	100[3]	100[3]

TABLE 35. (Cont/)

Proteolytic anaerobes

Expt.	Treatment	Age of silage (days)				
		1	2	3	8	180
8	Standard[1]	26	14		0.6	*260
	+ lactobacilli[2]	<0.23	<4.3		<100	<0.23
9	Standard[1]	<0.6	<0.6	<0.6	<0.6	<0.6
	+ lactobacilli[2]	100[3]	100[3]	100[3]	100[3]	100[3]
10	Standard[1]	<0.6	<0.6	0.6	<0.6	<0.6
	+ lactobacilli[2]	>430	100[3]	<100	100[3]	100[3]

1 Most probable numbers ~~per~~ 10^5

2 Count (most probable numbers) expressed as % of count of standard

3 Counts in treatment and standard both $<0.6 \times 10^5$

* Mould development occurred in this tube.

TABLE 36.

Effect of treatments on individual species

Figures represent the number of silages of each treatment in which a particular species was detected in dilutions of 1:12,000 or higher.

Treatment	Species								
	1	2	3	5	6	8	S	B	W
22°	3	1	-	-	-	-	2	3	-
40°	-	-	-	2	-	1	3	6	1
Uncut	7	2	1	-	3	-	4	7	-
Minced	-	2	1	-	-	-	-	-	1
+ water	10	4	-	-	-	1	1	6	-
Wilted	-	1	1	-	1	-	2	5	-
Wilted + water	5	3	1	-	-	-	1	6	1
+ lactobacilli	-	2	-	-	-	-	-	1	-
30°	5	5	-	-	1	-	4	6	1

1 , 2 etc: types 1, 2 etc.
S: Cl. sporogenes
B: Cl. bifermentans
W: Cl. welchii

however, have been responsible.

Evidence has been put forward by several investigators that wilting of green crops reduces the multiplication of anaerobes when the crops are ensiled (Introduction p. 16). It has also been suggested that an increase in the moisture content stimulates certain species (Introduction p. 16). A more detailed discussion of the whole problem will be given in a later section when the changes initiated by anaerobes in ensiled grass are examined.

The effect of inoculation with lactobacilli

The relevant data are presented in Table 35. Inoculation with lactobacilli resulted in low pH levels being reached more quickly than in other treatments (Table 30, p. 85). This may provide a complete explanation for the finding that the growth of anaerobes was inhibited. In experiment 10, where anaerobes were detected in dilutions of 1 : 12,000 or higher after one and two days, the pH level had fallen less rapidly than in other instances.

The effect of the treatments on individual species

The information available is presented in Table 36. This information is not complete as regards lactate fermenters, since identification was carried out in a proportion of cases only. No definite conclusions emerge from the data. The rare occurrence of proteolytic species in silage made from minced grass and grass plus lactobacilli is in all probability due to the low pH levels reached. It must be noted that the only

lactate/

TABLE 37.

Butyric acid content of silages in experiments 7 - 10

Expt.	Treatment	Butyric acid[1] 8 days	Butyric acid[1] 180 days
7	22°	3.3	21.0
	40°	10.9	14.8
	Uncut	14.5	37.5
	Minced	1.95	30.6
	+ water	27.0	59.2
	Wilted	0.05	4.0
	Wilted + water	16.5	45.5
	30°	9.5	24.6
8	22°	5.74	15.0
	40°	5.2	3.26
	Uncut	23.5	12.9
	Minced	17.7	9.8
	+ water	23.6	42.8
	Wilted	3.89	12.9
	Wilted + water	35.5	19.3
	+ lactobacilli	0	0.07
	30°	16.4	0 *
9	22°	0.11	5.7
	40°	0.14	0
	Uncut	0.73	-
	Minced	0.56	0
	+ water	7.26	7.15
	Wilted	5.2	0.1
	Wilted + water	0.12	3.6
	+ lactobacilli	0.79	1.1
	30°	0.11	3.6
10	22°	0.28	0.29*
	40°	1.8	3.6
	Uncut	0.25	3.5
	Minced	0.28	0
	+ water	1.87	0.8
	Wilted	1.08	1.7
	Wilted + water	5.52	1.5
	+ lactobacilli	0.59	0
	30°	0.97	5.6

1 ml. N acid per 100 g. grass dry matter

~~2 Age of silage in days~~

* Mould growth occurred in these tubes.

lactate attacking anaerobe so far identified as multiplying in the 40° treatment is type 5.

Comparison of the bacteriological and chemical results from experiments 7 - 10.

Butyric acid content

In the Introduction (p. 14) the agents which could be involved in the accumulation of butyric acid were discussed and reasons were given for the belief that only anaerobic micro-organisms are responsible for the production of this acid. The results of quantitative determinations of butyric acid should therefore be compared with the bacteriological data.

The quantities of butyric acid present in the silages after eight days and six months incubation are shown in Table 37. From Tables 45-8 in the appendix (p.106-9) it may be seen that anaerobes showed little or no multiplication during the fermentation occurring in the following treatments: experiment 7 - wilted; experiment 8 - + lactobacilli; experiment 9 - 40°, minced, wilted and + lactobacilli; experiment 10 - 22° and minced. Further, in these treatments very low values for butyric acid were obtained by chemical methods.

The bacteriological and chemical data suggest that it is lactate fermenters which are chiefly responsible for the production of butyric acid. Table 37 shows that the highest values for butyric acid were obtained when water was added to fresh or wilted grass in experiment 7, and that a high butyric acid/

TABLE 38.

Comparison of numbers of lactate fermenting anaerobes and butyric acid content in test-tube silages.

Expt.	Treatment	[1] Lactate fermenters					pH		[2] Butyric acid	
		1d	2d	3d	8d	180d	8d	180d	8d	180d
10	Uncut	<0.6	9	5.4	2.4	1.2	4.85	4.17	0.25	3.5
	+ water	140	9	9	<0.6	<0.6	4.39	4.00	1.87	0.8
	Wilted	0.6	14	14	9	<0.6	5.47	4.35	1.08	1.7
	Wilted + water	240	54	24	0.6	<0.6	4.41	3.98	5.52	1.5
	+ lactobacilli	56	5.6	<0.6	<0.6	<0.6	3.83	3.76	0.59	0
9	+ water	90	90	24	54	1.2	4.32	3.98	7.26	7.15
	Wilted + water	<0.6	900	12	24	3.6	5.09	4.20	0.12	3.6
7	30°	<0.6	18	48	120	9	5.74	5.26	9.5	24.6
	Uncut	<0.6	2.4	120	9	240	5.40	5.02	14.5	37.5
	+ water	16	32	160	72	16	4.74	4.74	27.0	59.2
	Wilted + water	1.2	<0.6	90	2.4	6.6	4.80	4.72	16.5	45.5

[1] 10^5 lactate attacking anaerobes per g. grass dry matter
[2] ml. N acid per 100 g. grass dry matter

acid content was also found in the minced grass of the same experiment. In experiment 8, large amounts of butyric acid accumulated when grass was ensiled with the addition of water. Further, in all these treatments numbers of proteolytic anaerobes remained low (Tables 45-6 p. 106-7). In the ensuing discussion, comparisons will therefore be made between the multiplication of lactate fermenting anaerobes and butyric acid content.

In several of the treatments in experiment 10 (Table 38), anaerobes initiated multiplication, yet only small amounts of butyric acid accumulated. Also, in two treatments in experiment 9, the numbers of lactate fermenters found were not significantly less than in silages of experiment 7 (Table 38), but the butyric acid content of the former was considerably lower. Speculations based on the work of Heinemann (1915), Rahn (1932), Foter & Rahn (1936) and the pH range suitable for the growth of anaerobes (Introduction p. 12) indicate that the low pH values reached may have been the cause of at least some of these unexpected findings. The above mentioned authors have shown that in bacterial cultures multiplication precedes the accumulation of the main bulk of the metabolic products. It appears possible that growth occurred under favourable pH conditions at the start of the ensilage process, but that all activity was then stopped by inhibitory pH levels. Such an explanation, however, does not fit the facts too well in some of the cases (experiment 10 - uncut and wilted; experiment 9 - wilted plus water).

In/

TABLE 39.

Comparison of numbers of lactate fermenting anaerobes and butyric acid content in silages of experiment 7

Treatment	Moisture content (%)	Lactate fermenters					pH		Butyric acid[4]	
		1d	2d	3d	8d	180d	8d	180d	8d	180d
30° [1]	78.9	<0.6	18	48	120	9	5.74	5.26	9.5	24.6
Uncut[2]	78.9	100[3]	13.3	250	7.5	2660	5.40	5.02	14.5	37.5
+ water[2]	86.4	>2660	170	330	60	170	4.74	4.74	27.0	59.2
Wilted + water[2]	78.8	> 200	< 3.3	180	2.0	73	4.80	4.72	16.5	45.5
Wilted[2]	65.6	100[3]	< 3.3	<1.2	0.5	<6.6	6.30	5.45	0.05	4.0

1 Counts of lactate fermenters given as 10^5 organisms per g. grass dry matter
2 Counts of lactate fermenters expressed as percentages of counts in 30° treatment
3 Counts in both treatment and standard $< 0.6 \times 10^5$
4 ml. N acid per 100 g. grass dry matter

In Table 39, the relevant data for several of the treatments in experiment 7 is presented. Numbers of anaerobes are given as percentages of numbers at 30°. The chemical results show considerable differences between the treatments. These differences have, however, no counterpart in the bacteriological data. Consideration of pH levels offers no explanation for this. Thus, the addition of water appears to promote the accumulation of butyric acid. Although the moisture contents of the wilted grass after the addition of water and the standard were almost identical, it is unlikely that as much water will be taken up after wilting as was lost before. The presence of "free" water as opposed to water held by protoplasmic colloids would seem to be the factor involved. It is of interest that the abstraction of water affected the accumulation of butyric acid in the opposite direction from its addition (wilted, Table 39).

The evidence shows that the correlation between butyric acid content and growth of the anaerobes detected is not good in some cases, and that in a proportion of these instances, the moisture content of the grass is involved. The scope of the work done has not been sufficient to provide information beyond that point. A further/short discussion of the problem will, however, be undertaken.

The explanation of the discrepancies between butyric acid content and numbers of anaerobes may lie in the fact that attention was concentrated on changes in the microflora occurring during the early stages of the fermentation. Important developments/

developments at some later period may have been missed. Again, little information has been obtained concerning the extent of formation of inactive endospores under different conditions of ensilage and the stage of the fermentation at which sporulation takes place.

The possibility that some species of importance has not been detected cannot be entirely dismissed. Possible weaknesses in the bacteriological techniques are that no media designed for the enummeration of saccharolytic organisms unable to ferment lactate were employed, and further, that incubation of all cultures was carried on at 37°. The incubation temperature of the silages in question was 30°. Although no anaerobes with a temperature maximum below 37° have been described in the literature, such organisms may exist (cf. genera Bacterium and Corynebacterium).

The mechanism whereby changes in moisture content affect the accumulation of butyric acid deserves investigation. An increase in the amount of free water may well result in the greater liberation of nutrients from plant cells. Conversely, wilting may lead to inadequate amounts of bacterial nutrients becoming available. The effect of wilting certainly cannot be explained in terms of inhibition by acidity. It may be that nutrients required in small amounts, e.g. growth factors, can become limiting factors to the activities of anaerobes in ensiled grass.

Finally, in several of the treatments of experiment 8, a considerable reduction of the butyric acid content occurred after/

TABLE 40.

Volatile base content, numbers of proteolytic anaerobes, and identity of the dominant types in test-tube silages

Expt.	Treatment	Volatile base[1]				Proteolytic anaerobes					
		8d	180d	Change from 0 to 8d	Change from 8 to 180d	3d		8d		180d	
						No.	D.S.	No.	D.S.	No.	D.S.
7	22°	12.1	25.3	+ 8.25	+ 13.2	5.6	B	26	S	90	S
	40°	11.5	18.2	+ 7.65	+ 6.7	0.6	B	0.6	S	<0.6	-
	Uncut	17.6	19.3	+13.75	+ 1.7	24	B	120	S	240	S
	Minced	13.7	14.8	+ 9.85	+ 1.1	<0.6	-	<0.6	-	<0.6	-
	+ water	8.5	21.2	+ 4.65	+ 12.7	<0.6	-	7.2	B	0.08	S
	Wilted	5.02	9.05	-	+ 4.03	1.4	B	1.4	B	0.6	S
	Wilted + water	7.1	7.7	-	+ 0.6	12	B	<0.6	-	<0.6	-
	30°	13.2	28.1	+ 9.35	+ 14.9	120	S	90	B &S	240	S
8	22°	9.7	14.5	+ 8.62	+ 4.8			26	B	<0.6	-
	40°	7.5	12.1	+ 6.42	+ 4.6			2.6	B	<0.6	-
	Uncut	8.6	6.4	+ 7.52	- 2.2			140	B	<0.6	-
	Minced	7.0	12.1	+ 5.92	+ 5.1			<0.6	-	<0.6	-
	+ water	8.2	30.2	+ 7.12	+ 22.0			1.4	B	<0.6	-
	Wilted	8.5	15.0	+ 5.67	+ 6.5			140	B	14	S
	Wilted + water	5.1	4.1	+ 2.27	- 1.0			<0.6	-	<0.6	-
	+ lactobacilli	6.5	5.4	+ 5.42	- 1.1			<0.6	-	<0.6	-
	30°	5.9	17.8*	+ 4.82	+ 11.9			0.6	B	260*	S*

No.: 10^5 proteolytic anaerobes per g. grass dry matter.
D.S.: Dominant species
[1]: ml. N alkali per 100 g. grass dry matter
*: Mould development occurred in these tubes.

B = Cl. bifermentans
S = Cl. sporogenes

TABLE 41.

Volatile base content, numbers of proteolytic anaerobes and identity of the dominant type in test tubes silages

Expt.	Treatment	Volatile base[1]				Proteolytic anaerobes					
		8d	180d	Change from 0 to 8d	Change from 8 to 180d	3d		8d		180d	
						No.	D.S.	No.	D.S.	No.	D.S.
9	22°	8.5	14.3	+ 1.8	+ 5.8	<0.6	-	<0.6	-	<0.6	-
	40°	4.5	15.2	- 2.2	+ 10.7	1.4	S	<0.6	-	<0.6	-
	Uncut	8.5	13.0	+ 1.8	+ 4.5	1.4	B	0.6	S	<0.6	-
	Minced	7.6	9.4	+ 0.9	+ 1.8	<0.6	-	<0.6	-	<0.6	-
	+ water	14.0	15.1	+ 7.3	+ 1.1	<0.6	-	1.2	S	<0.6	-
	Wilted	2.8	4.7	+ 2.11	+ 1.9	<0.6	-	<0.6	-	<0.6	-
	Wilted + water	10.9	12.0	+ 10.21	+ 1.1	<0.6	-	<0.6	-	<0.6	-
	+ lactobacilli	5.8	6.9	- 0.9	+ 1.1	<0.6	-	<0.6	-	<0.6	-
	~~Control~~ 30°	7.8	14.3	+ 1.1	+ 6.5	<0.6	-	<0.6	-	<0.6	-
10	22°	14.8	4.9*	+ 14.53	- 9.9	<0.6	-	<0.6	-	<0.6*	-*
	40°	9.9	9.9	+ 9.63	0	<0.6	-	<0.6	-	<0.6	-
	Uncut	13.2	8.2	+ 12.93	- 5.0	0.6	B	<0.6	-	<0.6	-
	Minced	7.7	4.6	+ 7.43	- 3.1	<0.6	-	<0.6	-	<0.6	-
	+ water	11.6	12.3	+ 11.33	+ 0.7	<0.6	-	<0.6	-	<0.6	-
	Wilted	8.9	6.4	+ 8.05	- 2.5	<0.6	-	<0.6	-	<0.6	-
	Wilted + water	14.1	9.6	+ 13.25	- 4.5	<0.6	-	0.6	B,S,	<0.6	-
	+ lactobacilli	8.3	4.9	+ 8.03	- 3.4	<0.6	-	<0.6	-	<0.6	-
	~~Control~~ 30°	13.5	10.4	+ 13.23	- 3.1	0.6	B	<0.6	-	<0.6	-

No: 10^5 proteolytic anaerobes per g. grass dry matter
D.S.: dominant species
1 : ml. N alkali per 100 g. grass dry matter
* : Mould development occurred in these tubes.

B = Cl. bifermentans
S = Cl. sporogenes

after eight days. The results might be taken to suggest that organisms capable of destroying butyric acid can at times proliferate in ensiled grass. No species capable of attacking butyric acid under the acid/anaerobic conditions prevailing in silage has yet, however, been described.

Volatile base content

An increase in the volatile base content of ensiled grass may be expected when the degradation of proteins and related compounds is carried beyond the amino-acid stage. The data relevant to this aspect of the problem are presented in Tables 40 and 41.

A considerable increase in the volatile base content had occurred in the majority of silages during the first eight days of incubation. The results, however, show no relation to the multiplication of anaerobes. In many of the silages in which an early increase in volatile base was found, proteolytic anaerobes could not be detected in dilutions of 1 : 12,000 (e.g. silages in experiment 10; wilted grass plus water in experiment 9; minced grass in experiment 7). The agents active during the first eight days appear to be of importance, as even in silages where the pH never fell below 5 (e.g. 22°, uncut and standard in experiment 7; see also Table 30 p.85), one-third or more of the amount of volatile base found after six months had already been produced by eight days. The plant cells rather than bacteria may be largely concerned in the formation of volatile base during the early stages of the fermentation.

In many silages, further accumulation of these bases occurred/

occurred between eight days and six months. In this period bacterial action might be presumed to be responsible. In silages where proteolytic anaerobes were active after the eighth day, as judged by counts in the selective medium, volatile bases accumulated. Comparable increases in such bases were, however, found in silages where only few or no proteolytic anaerobes could be detected, e.g. grass plus water in experiments 7 and 8. No evidence has been obtained that indicates the agents concerned in the formation of volatile base in these latter silages. Until the position is clarified no conclusion can be drawn about the action of anaerobes on proteins or their cleavage products. As a whole, the evidence suggests that this action in silage may be of less importance than has previously been thought to be the case (Schieblich, 1926).

It must further be pointed out that the volatile bases formed in silage may not invariably accumulate. Decreases after eight days incubation have been found in experiments 8 and 10. These decreases may have been due to a variation in the fermentations occurring in different tubes. If, on the other hand, volatile bases can undergo further changes, no interpretation of the data is possible with the information available.

Origin of the butyric acid formed

In certain of the experiments a good correlation was found to exist between the highest numbers reached by lactobacilli and the/

FIG. 11. CORRELATION OF THE PEAK COUNT OF LACTOBACILLI WITH LACTIC ACID CONTENT AFTER 6 MONTHS.

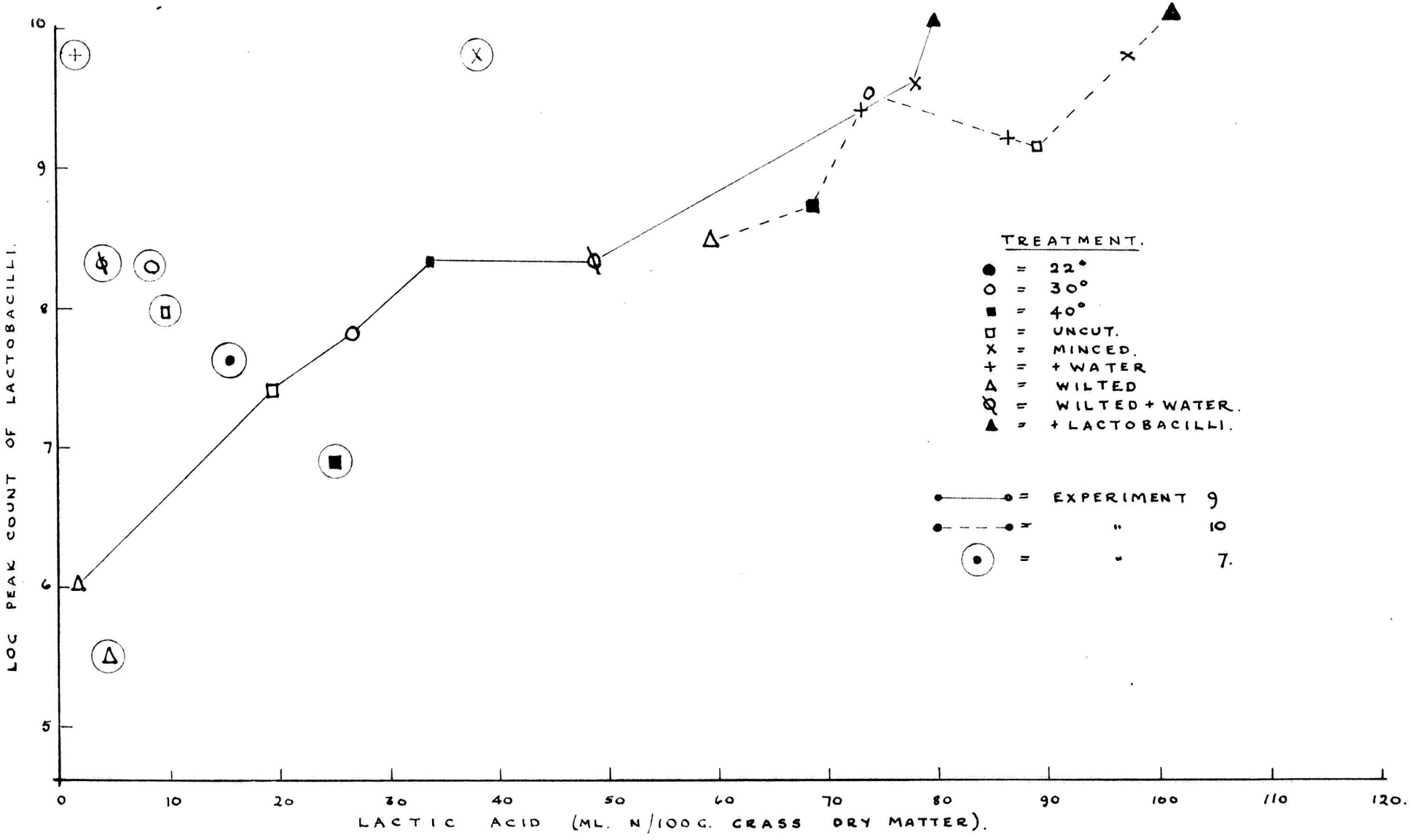
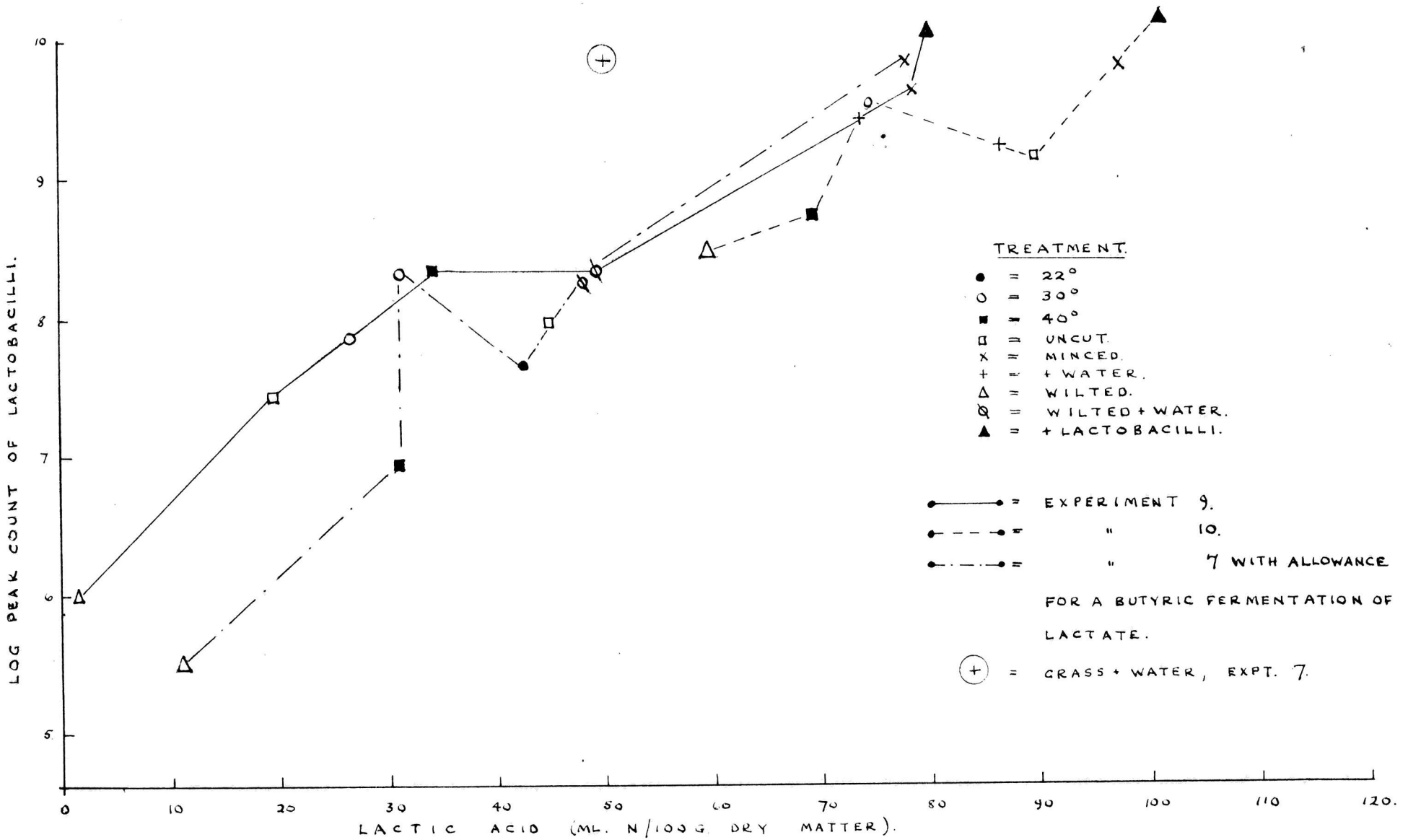


FIG. 12. CORRELATION OF THE PEAK COUNT OF LACTOBACILLI WITH LACTIC ACID CONTENT AFTER ALLOWANCE FOR A BUTYRIC FERMENTATION OF LACTATE.



the content of lactic acid after six months. This was the case in experiments 9 and 10 but not in experiment 7 (Fig. 11). (No correlation between counts of lactobacilli and lactic acid content appeared in experiment 8. Because of the incomplete data obtained and the unexplained reductions in butyric acid content (p. 92) this experiment will, however, be omitted from the discussion). In experiment 7, production of butyric acid was more extensive than in 9 or 10, and it appeared possible that the fermentation of lactate to butyrate was responsible for the lack of agreement between lactic acid content and the growth of organisms producing lactic acid.

Bhat & Barker (1947) have published data from which the amount of lactic acid utilised by Cl. lacto-acetophilum per mole of butyric acid formed can be obtained. Their figures were obtained from fermentations with pure cultures in the presence of acetate and also from enrichment cultures employing CO_2 as a hydrogen acceptor. Using this information, it was found that a butyric fermentation of lactate could explain the discrepancies in experiment 7 (Fig. 12). The lactic acid values shown in Fig. 12 have been corrected on the assumption that all the butyric acid formed after eight days originated from lactate and that the data obtained from pure culture fermentations is applicable. A much poorer fit was obtained when corrections were made on the basis of figures from the enrichment type of fermentation. It may be pointed out that considerable amounts of acetate were present in the silages of experiment 7.

TABLE 42.

Species of anaerobes isolated from two farm silages and the dilutions from which pure cultures were obtained

Species	No. of pure cultures obtained	Dilutions from which cultures were isolated
Type 1	7	$\frac{1}{12 \times 10^3}$, $\frac{1}{12 \times 10^4}$, $\frac{1}{12 \times 10^5}$,
" 3	7	$\frac{1}{12 \times 10^4}$, $\frac{1}{12 \times 10^5}$
" 4	2	$\frac{1}{12 \times 10^5}$
" 6	3	$\frac{1}{12 \times 10^4}$
" 7	1	$\frac{1}{12 \times 10^5}$
" 8	2	$\frac{1}{12 \times 10^4}$
" 9	2	$\frac{1}{12 \times 10^4}$, $\frac{1}{12 \times 10^5}$
Cl. sporogenes	15	$\frac{1}{12 \times 10^3}$, $\frac{1}{12 \times 10^4}$, $\frac{1}{12 \times 10^5}$
Cl. bifermentans	2	$\frac{1}{12 \times 10^3}$

Average pH values of samples: Grass + molasses - 5.5
Silorated grass - 5.0

Bacteriological results obtained from farm silages

Details of the preparation of these silages and the composition of the fresh grass are given on p. 33 and in Table 5. During the feeding of the silage to stock, samples were taken and prepared for bacteriological examination as described on p. 33 . Eight different layers in each of the two silos were sampled, and their content of anaerobes investigated as detailed on p. 57 et sequitur, using the two selective media. Only two replicate tubes were, however, inoculated from each dilution. As data from dilution counts with two replicates are difficult to interpret, the discussion will be confined to the species isolated and the dilutions in which different types were found (Table 42).

Lactate attacking and proteolytic anaerobes were present in high numbers. Type 3, found by van Beynum & Pette (1935 - 36, 1936) to be of common occurrence in silage, was one of the dominant species. Of interest is the presence of organisms of type 4. During the dissimilation of lactate by anaerobes of type 4, caproic acid is formed and this acid has been stated to occur in some silages (Virtanen & Miettinen, 1951). The diversity of the anaerobic microflora is worthy of note. Of the species incapable of attacking lactate or gelatin, types 6 and 8 have on occasion been isolated from the test-tube silages (Table 24, p.79). Types 4, 7 and 9 have, however, not been found in grass ensiled in test-tubes.

SUMMARY

The objects of the work described in this thesis have been (a) the development of methods suitable for the enumeration of vegetative cells as well as spores of the dominant species of anaerobes in ensiled grass, and (b) the investigation of the anaerobic micro-flora of grass silage by such methods.

As a preliminary to the development of the required techniques, the death of vegetative cells of Cl. butyricum during the preparation of dilutions, the ability of five commonly used reducing agents to prevent ingress of oxygen into media, and the toxicity of three of the reducing agents toward Cl. butyricum, Cl. acetobutylicum, Cl. welchii and Cl. sporogenes were studied.

The death of vegetative cells during the dilution procedure, using water as a diluent and taking no special precautions to prevent contact with oxygen, was found to be a negligible source of error. Thioglycollic acid, cysteine and sodium formaldehyde sulphoxylate were more effective in preventing the oxidation of media than ascorbic acid or glucose evolué. Cysteine, in concentrations of up to 0.5% w/v showed no inhibitory effect towards the four test organisms. Thioglycollic acid and sodium formaldehyde sulphoxylate each proved toxic to two of the four species, unless added in low concentrations. By the use of reducing agents, growth of anaerobes was obtained in shallow layers of agar in 50 ml. conical flasks and in Petri dishes.

The identity of the dominant anaerobes in ensiled grass was investigated by primary culture of dilutions of silage in corn-liver mash, followed by transfer to a medium in which many species/

species form spores readily, pasteurisation and isolation on non-selective media in that order. All the cultures obtained either had the ability to ferment lactate in the presence of acetate or else attacked proteins or their cleavage products.

Media containing lactate, acetate and small quantities of yeast autolysate supported quantitative growth of lactate fermenting anaerobes. In such media, lactate-attacking organisms in mixed or pure culture could be recognised through the gas production and changes in pH level resulting from the fermentation. A dilution method, using a semi-solid lactate - acetate - yeast autolysate agar is therefore suitable for the enumeration of these types.

Proteolytic anaerobes were able to initiate growth from small inocula in media containing proteins and their cleavage products but no added carbohydrates. Further, among the micro-organisms tested, proteolytic anaerobes alone produced extensive decomposition in such media when oxygen was excluded. Vegetative cells and spores of the anaerobes in question, when contained in mixed populations, can be enumerated by a dilution technique employing a gelatin - peptone - yeast autolysate medium and testing for H_2S, NH_3 and indole production and gelatin liquefaction.

The value of the methods evolved lies in the fact that greater confidence can be placed in the results than was possible before. When spores alone are counted, the data provide at the most a rough indication of the extent of proliferation. Since sporulation does not occur during active growth, the/

the course of multiplication cannot be followed by counting spores. For a better understanding of the chemical changes initiated by anaerobes, information concerning vegetative cells is essential due to the inactive nature of spores.

Grass silage was prepared in large test-tubes. Changes occurring during ensilage were followed by examining tubes at intervals. The lactate attacking anaerobes detected most frequently were Cl. lacto-acetophilum, Cl. tyrobutyricum and a species not previously described as occurring in ensiled fodder. The dominant proteolytic anaerobes were Cl. bifermentans and Cl. sporogenes. In a few silages, saccharolytic species incapable of attacking lactate had multiplied.

Cl. bifermentans and two of the lactate fermenting species initiated multiplication within the first two days after ensilage. At low pH levels, the growth of all types was inhibited. Differences in the degree of chopping of the grass prior to ensilage and inoculation with lactobacilli affected the anaerobic micro-flora when these treatments promoted the production of acid. A marked inhibition of the growth of anaerobes occurred when grass was wilted, and this effect could not be explained in terms of inhibition by acidity. A temperature of 40° during the ensilage process appeared to depress the growth of lactate fermenters, but not through the attainment of low pH levels.

Comparison of bacteriological and chemical data showed that/

that agreement between butyric acid content and the multiplication of the anaerobes detected is not good in all cases. The moisture content of the grass seems to be able to affect this correlation, increases in the mositure content apparently stimulating butyric acid production. Indications were further obtained that the part played by anaerobes in the accumulation of volatile bases may not be as great as has at times been suggested and that lactic acid is an important substrate for the butyric fermentation.

TABLE 43.

Penetration of oxygen into methylene blue-agar containing reducing agents

Figures represent the depth of the oxidised layer in cm.
Depth of medium in tubes - 4.5 cm.

Thioglycollic acid

Concentration[1]	Days of storage							
	2/3	3	5	9	11	13	15	25
0	2.5	4.5						
0.1	0.55	0.9	1.0	1.25	1.5	1.8	1.95	4.5
0.2	0.35	0.55	0.7	1.0	1.0	1.1	1.2	1.95
0.3	0.2	0.4	0.6	0.7	0.75	0.8	0.85	1.55
0.4	0.2	0.2	0.2	0.4	0.45	0.5	0.5	0.9
0.5	0.2	0.25	0.2	0.3	0.35	0.35	0.4	0.7

1 = $\%\frac{v}{v}$

TABLE 43. Cont/.

Na formaldehyde sulphoxylate

Concentration[1]	Days of storage							
	2/3	3	5	9	11	13	15	25
0	2.4	4.5						
0.2	0.4	1.15	1.4	2.25	2.7	3.4	4.5	
0.4	0.15	0.45	0.55	0.8	0.85	-	1.0	1.55

1 = % w/v

At concentrations greater than 0.4% methylene blue is permanently decolourised.

Cysteine Hydrochloride

Concentration[1]	Days of Storage							
	1/4	2	3	5	7	10	13	16
0	1.6	4.0	4.5					
0.1	0.55	0.95	1.1	1.25	1.5	1.75	2.2	2.85
0.2	0.4	0.6	0.7	0.9	1.0	1.1	1.4	1.85
0.3	0.4	0.45	0.5	0.6	0.7	0.75	0.95	1.15
0.4	0.3	0.45	0.5	0.55	0.6	0.7	0.75	0.8
0.5	0.25	0.4	0.4	0.4	0.45	0.55	0.6	0.7

1 = % w/v

TABLE 43. Cont./

Ascorbic acid

Concentration[1]	Days of storage				
	1/4	2	3	5	8
0	1.55	3.9	4.5		
0.1	0.65	1.8	1.95	2.3	2.9
0.2	0.55	1.75	2.0	2.55	3.1
0.3	0.8	1.9	2.0	2.5	3.0
0.4	0.9	1.75	1.95	2.6	3.05
0.5	0.8	2.0	2.1	2.55	3.1

1 = % ⩽

Glucose evolué

Concentration[1]	Days of storage				
	2/3	3	5	9	11
0	2.7	4.5			
0.2	1.95	3.15	3.6	4.3	4.5
0.4	1.9	2.9	3.35	3.9	4.5
0.6	1.55	2.6	3.0	3.45	4.1
0.8	1.6	2.6	2.85	3.2	3.6
1.0	1.55	2.4	2.6	3.05	3.35

1 = % ⩽

TABLE 44.

The origin and identity of pure cultures obtained after primary cultivation in non-selective media.

Experiment	Age of silage (days)	pH	Butyric acid[1]	Types and No. of strains isolated (in brackets)	Dilutions from which strains were isolated
3 a	0.25 - 8	5.90[2]	0.1[2]	None	
b	0.25 - 8	5.68[2]	0.2[2]	None	
c	0.25	6.34	1.78	Type 2 (1)	$1/10^2$
	1.5	5.84	9.26	Type 1 (2) & Type 2 (3)	$1/10^5$, $1/10^6$, $1/10^7$
	2	5.46	22.3	Type 1 (1) & Type 2 (2)	$1/10^6$, $1/10^7$
	8	5.64	36.4	Type 2 (2)	$1/10^4$, $1/10^6$
4 a	1	6.68	2.9	Type 1 (2) & Type 2 (2)	$1/10^5$
	3	5.3	0.65	Type 1 (3) & Type 2 (1)	$1/10^5$, $1/10^6$, $1/10^7$
	8	4.6	-	Type 1 (5)	$1/10^5$, $1/10^6$
c	1	6.09	0.74	Type 2 (1)	$1/10^5$
5 a	9	5.3	13.8	Type 2 (1) Cl. sporogenes (2), & Cl. bifermentans (1)	$1/10^3$, $1/10^4$
b	1.25 - 9	5.94[2]	5.02[2]	None	
c	1.25 - 9	6.48[2]	0.42[2]	None	
6 a	0	-	-	Type 1 (1)	1/10
	2	6.4	0	Cl. welchii (1)	$1/10^4$

TABLE 44. Cont./

Experiment	Age of silage (days)	pH	Butyric acid	Types and No. of strains isolated	Dilutions from which strains were isolated
6b	0	-	-	Cl. welchii (1)	1/10
	2 - 14	5.5[2]	0[2]	None	
A 1	110	-	-	Cl. sporogenes (1)	$1/10^3$
2	140	-	-	Cl. sporogenes (2)	$1/10^3$
Farm silage					
X	140			Type 2(1), Type 3(1) & Cl. welchii (1)	$1/10^4$
Y	140			Type 1(1) & Type 3(2)	$1/10^3$, $1/10^5$

1 ml. N butyric acid per 100 g. grass dry matter
2 after longest period of storage

TABLE 45.

Bacteriological results obtained from Experiment 7.

Treatment	Age of Silage (Days)														
	1			2			3			8			180		
	L	P	O	L	P	O	L	P	O	L	P	O	L	P	O
22°	<0.6	<0.6	-	<0.6	1.3	-	0.06	5.6	-	14	26	-	250	90	-
Species present	-	-		-	B(i)		-	B		-	S		1(i)	S(i)	
40°	5.6	140	-	140	56	-	5.6	0.6	-	<0.6	0.6	-	<0.6	<0.6	1
Species present	-	B		-	B(i)		-	B		-	S		-	-	8(i)
Uncut	<0.6	2.4	1	2.4	24	-	120	24	-	9	120	-	240	240	-
Species present	-	B	6(i)	-	S		-	B		-	S		1(i),3(i)	S(i)	
Minced	14	<0.6	-	25	<0.6	-	260	<0.6	-	<0.6	<0.6	-	26	<0.6	-
Species present	-	-		-	-		-	-		-	-		3(i)	-	
+ water	16	<0.6	-	32	1.6	-	160	<0.6	-	72	7.2	-	16	0.08	-
Species present	-	-		-	B(i)		-	-		-	B		1(i)	S(i)	
Wilted	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	1.4	-	0.6	1.4	-	<0.6	0.6	-
Species present	-	-		-	-		-	B		-	B		-	S(i)	
Wilted + water	1.2	2.4	-	<0.6	12	-	90	12	-	2.4	<0.6	-	6.6	<0.6	-
Species present	-	B		-	B		-	B		-	-		1(i)	-	
30°	<0.6	<0.6	-	18	24	-	48	120	-	120	90	-	9	240	-
Species present	-	-		2(i)	B(i)		-	S			S,B		1	S(i)	

L : 10^5 lactate fermenters per g. grass dry matter (Most probable numbers)
P : 10^5 proteolytic anaerobes per g. grass dry matter (Most probable numbers)
O : Highest dilution (wet silage) in which anaerobes other than L or P were found.
(i): pure culture isolated.

B : Cl. bifermentans
S : Cl. sporogenes
1,2 etc. : types 1,2 etc.

TABLE 46.

Bacteriological results obtained from experiment 8.

Treatment	Age of Silage (days)														
	1			2			3			8			180		
	L	P	0	L	P	0	L	P	0	L	P	0	L	P	0
22°	<0.06	<0.06	-	<0.6	<0.6						26		26	<0.6	-
Species present	-	-		-	-						B		1(i)	-	
40°	26	140	-	<0.6	0.6						2.6		<0.6	<0.6	-
Species present	-	B & W(i)		-	S						B		-	-	
Uncut	0.3	<0.6	-	<0.6	14						140		260	<0.6	-
Species present	1(i)	-		-	B(i)						B		1(i)	-	
Minced	5.6	<0.06	-	-	140						<0.6		<0.6	<0.6	-
Species present	2(i)	-			W(i)						-		-	-	
+ water	6.0	5.6	~~1 12x10⁵~~	6.0	<0.6						1.4		26	<0.6	-
Species present	1(i),2(i)	B(i)	8	1(i)	-						B		1(i)	-	
Wilted	<0.06	0.2	-	<0.6	1.4						140		5.6	14	~~1 12x10⁵~~
Species present	-	B		-	B						B		-	S	6(i)
Wilted + water	0.06	13	-		14						<0.6		450	<0.6	-
Species present	-	B(i),W(i)			B(i)						-		1(i)	-	
+ lactobacilli	<0.06	<0.06	-	<0.6	<0.6						<0.6		<0.6	<0.6	-
Species present	-	-		-	-								-	-	
30°	0.1	26	-		14						0.6		*2600	*260	-
Species present	-	B(i),W(i)			B						B		1(i)	S(i)	

L : 10^5 lactate fermenters per g. grass dry matter (most probable numbers)
P : 10^5 proteolytic anaerobes per g. grass dry matter (most probable numbers)
0 : highest dilution in which anaerobes other than L or P were found
(i): pure culture isolated
* : mould development occurred in this tube.

B : Cl. bifermentans
S : Cl. sporogenes
W : Cl. welchii
1,2 etc.: types 1, 2 etc.

TABLE 47.

Bacteriological results obtained from experiment 9

Treatment	Age of silage (days)														
	1			2			3			8			180		
	L	P	O	L	P	O	L	P	O	L	P	O	L	P	O
22°	<0.06	<0.06	-	<0.6	<0.6	-	0.1	<0.6	-	0.1	<0.6	-	140	<0.6	-
Species present	-	-		-	-		2(i)	-		-	-		1	-	
40°	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	1.4	-	<0.6	<0.6	-	<0.6	<0.6	-
Species present	-	-		-	-		-	S		-	-		-	-	
Uncut	<0.6	<0.6	-	<0.6	<0.6	-	1.4	1.4	1 / ~~12x10⁴~~	2.6	0.6	1 / ~~12x10⁴~~	14	<0.6	-
Species present	-	-		-	-		1	B	6(i)	-	S	6(i)	1	-	
Minced	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.06	<0.06	
Species present	-	-		-	-		-	-		-	-		-	-	
+ water	90	<0.6		90	<0.6		24	<0.6		54	1.2		1.2	<0.6	
Species present	1(i), 2(i)	-		1(i)	-		1(i)	-		1(i)	S		1	-	
Wilted	<0.06	<0.06	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	
Species present	-	-		-	-		-	-		-	-		-	-	
Wilted + water	<0.6	<0.6		900	<0.6		12	<0.6		24	<0.6		3.6	<0.6	
Species present	-	-		2(i),1	-		2(i)	-		1(i)	-		1	-	
+ lactobacilli	<0.6	<0.6	-	<0.06	<0.06	-	<0.6	<0.6	-	<0.06	<0.6	-	<0.06	<0.06	-
Species present	-	-		-	-		-	-		-	-		-	-	
30°	<0.6	<0.6		1.4	<0.6	1 / ~~12x10⁵~~	2.3	<0.6		1.4	<0.6		0.06	<0.6	
Species present	-	-		2(i)	-	6(i)	2(i),1	-		1(i)	-		-	-	

L : 10^5 lactate fermenters per g. grass dry matter (most probable numbers).
P : 10^5 proteolytic anaerobes per g. grass dry matter (most probable numbers)
O : highest dilution (wet silage) in which anaerobes other than L or P were found

B : Cl. bifermentans
S : Cl. sporogenes
(i) : pure culture isolated
1, 2 etc.: types 1, 2 etc.

TABLE 48.

Bacteriological results obtained from experiment 10.

Treatment	Age of Silage (days)														
	1			2			3			8			180		
	L	P	O	L	P	O	L	P	O	L	P	O	L	P	O
22°	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	*<0.6	*<0.6	-
Species present	-	-		-	-		-	-		-	-		-	-	
40°	2.6	0.6	-	2.6	<0.6	-	2.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-
Species present	-	B		5(i)	-		5(i)	-		-	-		-	-	
Uncut	<0.6	<0.6	-	9	0.6	-	5.4	0.6	-	2.4	<0.6	-	1.2	<0.6	-
Species present	-	-		2(i)	B		2(i)	B		1(i)	-		1	-	
Minced	0.06	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.06	<0.06	-
Species present	2(i)	-		-	-		-	-		-	-		-	-	
+ water	140	2.6		9	1.4	-	9	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-
Species present	1	B		2(i)	B		2(i)	-		-	-		-	-	
Wilted	0.6	<0.6		14	<0.6	-	14	<0.6	-	9	<0.6	-	<0.6	<0.6	-
Species present	-	-		-	-		2(i)	-		3(i)	-		-	-	
Wilted + water	240	<0.6		54	<0.6	-	24	<0.6	-	0.6	0.6	-	<0.6	<0.6	-
Species present	-	-		-	-		2(i)	-		3(i)	B(i),S(i)		-	-	
+ lactobacilli	56	2.6		5.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.6	-	<0.6	<0.06	-
Species present	2(i)	B		2(i)	-		-	-		-	-		-	-	
30°	26	<0.6		5.6	<0.6	-	0.06	0.6	-	0.06	<0.6	-	<0.6	<0.6	-
Species present	2(i)	-		-	-		2(i)	B		1	-		-	-	

L : 10^5 lactate fermenters per g. grass dry matter (most probable numbers)
P : 10^5 proteolytic anaerobes per g. grass dry matter (most probable numbers)
O : highest dilution in which anaerobes other than L or P were found.
* : mould development occurred in this tube

B : Cl. bifermentans
S : Cl. sporogenes
1, 2 etc. : types 1, 2 etc.
(i) : pure culture isolated

Mineral supplement

Solution A -	0.08%	K_2HPO_4
	0.02%	KH_2PO_4
Solution B -	0.02%	$MgSO_4 . 7H_2O$
	0.01%	NaCl
	0.05%	$FeSO_4 . 7H_2O$
	0.0002%	$MnSO_4 . 4H_2O$
Solution C -	0.0002%	$Na_2MoO_4 . 2H_2O$

1 ml. of solutions C, B and A were added, in that order, per 100 ml. of medium.

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